

Where now with British energy?

The ending of a year-long miners' strike in Britain, and of a nuclear inquiry begun twice as long ago, will prompt reappraisal of fuel policy. The market matters most.

By ironical coincidence, the year-long British miners' strike and the public inquiry into the proposal to build a nuclear reactor in Suffolk are petering out at the same time. This week or next, half of the 180,000 British miners will have broken ranks and gone back to work, after months of heroic but appalling self-deprivation. And with counsel for the Central Electricity Generating Board, the national nationalized electricity utility, more than a fortnight launched on his final submission to the inspector charged with recommending whether Britain in general and the village of Sizewell in particular can safely house a pressurized-water reactor, that longer-running spectacle must also soon end. Within a year or so, the Sizewell inspector will have made his recommendation along predictable lines (see *Nature* 300, 672; 1982), these will have been further qualified by the British Department of Energy and the electricity board will be able to begin fulfilling an ambition first declared in 1972. The British should count themselves lucky that there has been oil in the North Sea to allow the luxury of these two serious interruptions of other sources of energy.

The Sizewell inquiry has been the more comical. Part of the explanation for the time that has been taken is the inspector's decision, at the outset of the proceedings, that documents could be accepted in evidence only if they were first read aloud, which has revealed many distinguished engineers and administrators to be only passable at public recitation. The scope of the inquiry, the most serious cause of the length of the proceedings, has raised questions about the constitutional status of public inquiries as such (see *Nature* 301, 100; 1983). That the people directly affected (and usually inconvenienced) by major developments should have an opportunity to demand an explanation from those responsible cannot be denied in democratic societies. But the Sizewell inquiry was unfortunately also given a licence to consider not merely the practical objections of those who do not want to see nuclear power stations in their backyards, or those who argue that less amenity would be sacrificed if they were put elsewhere, but the general questions of whether nuclear power in countries such as Britain is economically necessary and whether nuclear power as such contributes to the proliferation of nuclear weapons and the particular questions of whether pressurized water reactors are safer and more economical than other kinds of thermal reactors. The inspector, having listened to opinions of these questions for more than two years, will give his own opinions, which will carry what is called "weight" but will otherwise be irrelevant.

Promise

The mistake at Sizewell should now be plain. In Britain as in other democratic countries, the government consists of an executive and a legislature; the one proposes, the other disposes. (In Britain, there is a tendency to suppose the first synonymous with the whole.) The British government has also (since 1948) owned the machines that generate electricity but has delegated their operation, maintenance and replacement to the Central Electricity Generating Board (together with its congeners in Scotland and Northern Ireland), which is supposed to function as a commercial organization, earning a return on capital employed, making profits and otherwise behaving as if it were a corporation. So by what right, the managers of these enterprises may ask, has their decision to build nuclear rather than conventional power

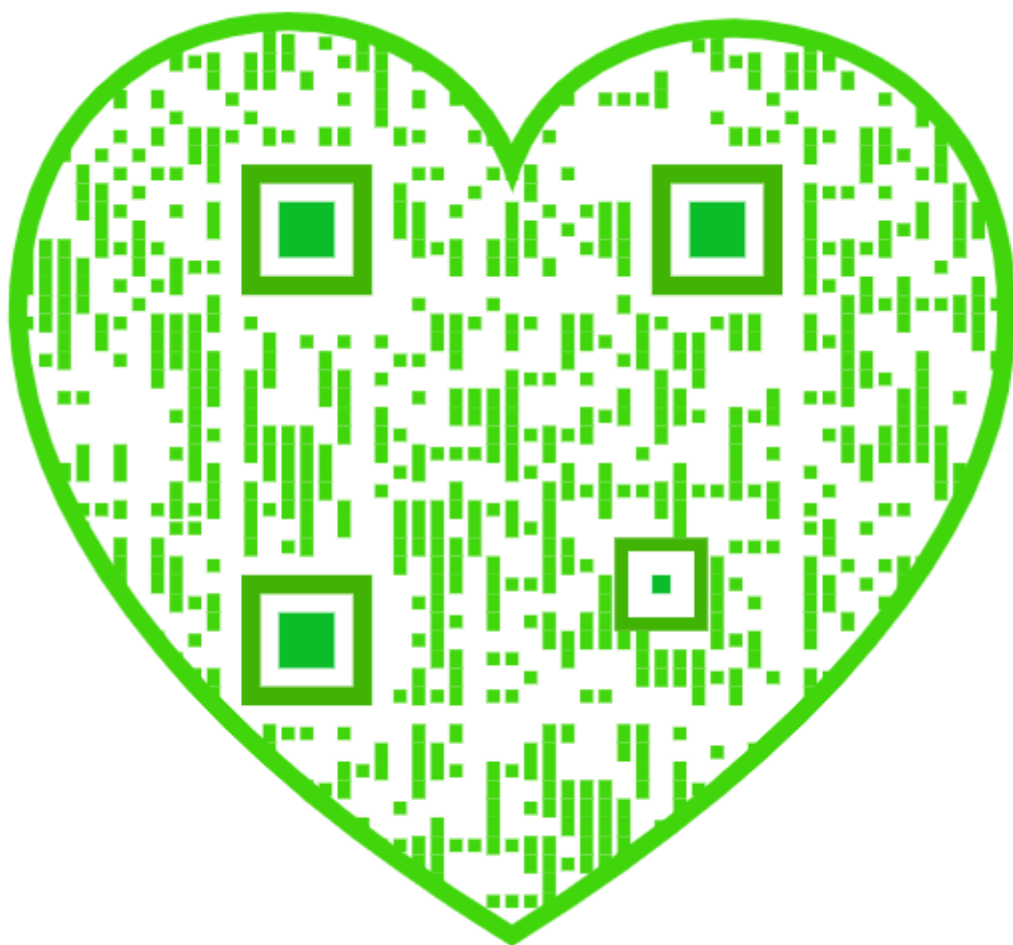
stations been put out to a public inquiry? The answer, that all nuclear power stations carry novel risks, and that the risks of pressurized-water reactors are literally novel to Britain, justifies special treatment but not the proceedings of the past two years. And whatever the inspector at Sizewell recommends, it will be for the executive to decide whether or not this course of development is compatible with public safety — and for the legislature to replace the administration if it makes a hash of its decision. So why could this not have been done five years ago, when the present British government first came to office with a plan for building nuclear power stations? Because it chose to be bound by its predecessor's promise that there would be a "generic" inquiry into pressurized-water reactors before a single one was built. It could, and should, have chosen otherwise.

Economics

The miners' strike is an even more serious matter. The British coal industry reached its peak in the 1920s, when close on a million miners produced 250 million tons of coal a year. Ever since, the industry has been in decline, the victim of geology (thin seams, for example) and economic competition, latterly from oil. While productivity has increased steadily over the decades, there have been several occasions when the labour force has been too small to match demand for coal, but more recently there has been a surplus. The issue underlying the strike has been the nationalized coal industry's ambition to concentrate production on economic pits and to reduce total production (by some four per cent). For practical purposes, the industry has only one important customer, the nationalized electricity industry (that which wants to build a single pressurized-water reactor). So one of the arguments between the miners and their employers that have soured the past twelve months could have been simply resolved by asking how much of present coal production would yield electricity costing more than that from a pressurized-water reactor. Another, the miners' insistence that jobs in the mining industry should not be lost, is incapable of resolution within a rational economic framework and is in any case belied by the historical flight from coalmining into more congenial jobs.

The difficulty, and the most bitter legacy of the past year's strike, is that neither the employers nor the government have been sufficiently sensitive to the plight of the mining communities likely to be left high and dry by the planned contraction of the industry. Britain already has too many of these abandoned communities, left over from past contractions, in which the disappearance of the major source of employment has been followed by the erosion of the social institutions which alone might help people physically to escape from an environment that can no longer support them. The British government (both halves of it) had better bend its mind to the imaginative search for solutions to these problems before more social damage is done.

The urge to celebrate the end of the strike and of the Sizewell inquiry by producing another "fuel policy" should however be resisted. During the past half-century, British governments of all colours have repeatedly embarked on attempts to foretell the future mix of fuels in the energy producing industries. The justification has been that forecasts of this kind are the only means by which the scale of investment in the nationalized industries can be planned most advantageously, which is another way of



saying that these plans have been attempts to balance the competing demands on public funds by the several still nationalized industries. These plans have regularly been falsified by changing technology (in energy conservation, for example) as well as by the changing economic environment in which countries such as Britain operate. The miners' strike has been marked by frequent references to a document called *Plan for Coal*, agreed in mid-1974 between the government, the industry and the trades unions as a prospectus for at least the following decade. Like other attempts to plan for an unpredictable future, *Plan for Coal* now seems like an attempt to make water run uphill. The intention then was that coal production should be at least 135 million tonnes by now (1985), well in excess of the 120 million tons of coal from all sources that over-saturated the market in 1982-83. The government's difficulty is that the plan is considered by the miners as a kind of treaty, for that is how it was arrived at.

For the future, there is only one viable fuel policy for Britain, that people's use of alternative fuels should be decided in the marketplace. Much of the peculiarly British difficulty is that the marketplace is dominated by public monopolies, the coal industry itself but also its chief customers. While there is no reason to suspect that these industries neglect the quasi-commercial ground-rules laid down by the government, the rules are more like abstract approximations to commercial reality than commercial realities themselves. The rate of interest paid on capital costs, whether of nuclear power stations or new coal mines, are settled by the British Treasury, not by the cautious lenders inhabiting bond-markets elsewhere. Similarly, profit targets are notional, and are not determined by the hurly-burly of real bargaining between sellers and buyers. What the British government needs is a better set of approximations to commercial reality, for which reason regulation of the import or export of fuels should be abandoned. Allowing or even encouraging the production of both coal and electricity outside the frameworks of the nationalized industries would also help. Selling off the coal industry, or parts of it, to private industry would further help to engender economic realism, but might also be the trigger for another strike. □

Anglo-Americanisms

Mrs Margaret Thatcher seems unusually to have been holding her tongue last week in Washington.

MRS Margaret Thatcher's visit to Washington last week seems to have been as prone to misunderstanding as most other occasions when straight talk is compromised by politeness. Nobody except the two people concerned can know what the British prime minister said privately to President Reagan, but there are at least two respects in which her message to the US Congress differed from what her constituents would have had her say. Both on star wars, otherwise known as the Strategic Defense Initiative, and on the relationship between the European and the US economies, Mrs Thatcher pulled her punches. Here is what she should have said.

First, on star wars, Mrs Thatcher passed up a splendid opportunity for clarifying the British view and, to a lesser extent, that of other Western European governments on this important issue. The general belief that anything more sophisticated than terminal defence in the old-fashioned 1970s version will be impractical is as widely shared as in the United States. There is nevertheless support for the notion that those who believe a layered defence against ballistic missiles is attainable should be given the benefit of the doubt and allowed to put their money where their mouths are, but that there can be no serious attempt to test such a system, let alone deploy it, without the agreement of the Soviet Union, with which the United States signed the Anti-Ballistic Missile (ABM) Treaty in 1972. On her return from her previous trip to Washington in December, Mrs Thatcher gave people to understand that the last point had been established during her conversations with the president. Fair play, last week she referred again to the need that no system of defence against missiles could be installed without negotiation, but failed to win

the endorsement of this view that would have been expected. She might have forced the issue by making more of her understanding of what the US administration intends, making it plainer than she did that her support for star wars, like the acquiescence of a proportion, probably a minority, of her compatriots, is conditional on that. For a woman who prides herself on plain talking, this is an awkward ambiguity to have left around.

The issue is far from being academic. The ABM treaty is based on the assumption that strategic stability requires that each superpower should continue to be hostage to the other's nuclear forces (and on the subsidiary assumption that building effective defences would be ruinously expensive). This is why the treaty allowed only the deployment of anti-ballistic missiles around each seat of government and one field of land-based rocket launchers. While it may be argued (and is in Washington) that, in the past decade, strategic missiles have been made more accurate and thus capable of tempting one side or the other into a pre-emptive strike against the other's retaliatory force, neither superpower can unilaterally change the rules of the strategic relationship without discomfiting the other. All this, no doubt, will be made clear on 12 March, when the United States and the Soviet Union are due to open strategic talks at Geneva. To pretend that the development of star wars is entirely unconstrained by past commitments is disingenuous, and can only lead to trouble, perhaps even the collapse of this new round of talks. Mrs Thatcher should have used the opportunity to make this crystal-clear.

She might also have talked more openly about the problems of the European economies or, as the Europeans see it, about the problems of the US economy and the US administration's management thereof. The dramatic strength of the dollar in the past year is a surprise, but otherwise merely a proof that people outside the United States are more attracted by the health of the domestic US economy than they are offended by the size of the US external trade deficit. Trading partners of the United States could and should live with this imbalance, which has allowed them to increase exports (to the United States) and, in the process, to see their industries become more competitive. What hurts is not the strength of the dollar but the generally high interest rates which are a consequence of the huge federal deficit. Europeans and others, by lending to the United States, are accumulating substantial stakes in the future of US enterprise, while US taxpayers will be helping to pay European pensions for many years to come, but the high cost of capital is a serious drag on the pace of European growth. That is one complaint.

Two other worries deserved a hearing at last week's talks, of which the most immediate is that the United States cannot, in this delicately unbalanced relationship with the rest of the world, seriously think of changing the rules of international trade. To its credit, the administration has been urging in the past few weeks that there should be another round of talks under the general rubric of the General Agreement on Tariffs and Trade, but the world knows that Congress is awash with special pleading on behalf of sections of US industry suffering from the competition of foreign goods cheapened by the dollar's strength. Where better to have uttered a warning on this subject than at a joint meeting of both houses of the US Congress?

The more distant but potentially more serious worry is that the present imbalance between the United States and Europe may one day be unwound catastrophically, by a sudden collapse of confidence in the value of dollar investments. While for the time being there is hardly a cloud on the horizon, it has quickly been forgotten that there have been several occasions in the past few years when there has been general alarm about the stability of important financial institutions in the United States. Continental Illinois, the Chicago bank, collapsed last year because of its domestic lending policy, and could have been the first in a line of dominoes. But the point at which Europeans and others stop lending to the United States will almost certainly be determined by an event quite different and, for the time being unexpected. That is when the turnaround will come, to everybody's discomfiture. Mrs Thatcher should have made that point as well. □

AIDS

US administration's parsimony criticized

Washington

THE Reagan administration has consistently refused to seek adequate support for research into acquired immune deficiency syndrome (AIDS), according to a study by the Congress's Office of Technology Assessment (OTA) released last week.

While praising the government scientists who have carried out much of the research so far on AIDS, the OTA study chastised the Department of Health and Human Services for repeatedly rebuffing the recom-

such test, but has not announced a firm date. In the meantime, criticism of the impending implementation of the tests, which detect antibody to the AIDS virus, is threatening to boil over. A number of blood-bank officials and clinical physicians worry about being placed in the impossible position of having to explain to patients the unknown significance of a positive test result. And homosexual organizations worry that test results, especially if they became part of a patient's permanent medical records,

	Internal PHS request	Official administration request	Congressional appropriation
1986	not available	\$86 million	not available
1985	\$91 million	\$61 million	\$97 million
1984	\$60 million	\$40 million	\$61 million
1983	not available	-	\$29 million
1982	-	-	\$6 million

Source: *Review of the Public Health Services's response to AIDS*, Office of Technology Assessment Technical Memorandum OTA-TM-H-24, February 1985.

mendations of its own Public Health Service (PHS) agencies for emergency increases in AIDS research funds. For the past three years, Congress has provided additional support in spite of objections from the administration (see table). The administration is proposing to hold the AIDS research budget for next year at the current level of \$86 million.

The official administration line continues to be that the Public Health Service can handle the AIDS crisis by shifting funds and personnel from other areas. The Centers for Disease Control (CDC) have cut back work on hepatitis B and sexually-transmitted diseases in order to supplement research on AIDS, the Food and Drug Administration has cut back work on hepatitis vaccine and the National Institutes of Health (NIH) have shifted resources mainly from cancer research.

The OTA study points to several indicators that support remains inadequate, despite congressional action (based on leaked documents showing the original budget plans submitted by the PHS agencies) to appropriate additional monies: the study notes continued rivalries between PHS agencies, notably NIH and CDC, for funding; recommendations by two National Cancer Institute advisory committees that support must be increased; and the privately voiced opinions of "numerous" federal and non-federal scientists that current efforts are inadequate.

The study also criticized the fanfare surrounding Secretary of Health and Human Services Margaret Heckler's announcement last April that the causative agent of AIDS had been isolated and that a blood test would be on the market within six months. FDA says it will shortly license the first

could be used as grounds for denying life or health insurance or employment; furthermore, given the high level of exposure to the virus among male homosexuals, the fear is that employers could use the test simply as an indicator of homosexuality.

The National Gay Task Force has recommended that the test be used only for screening the blood supply and for clinical research; it has urged its members not to take the test. Another homosexual rights organization, the Lambda Legal Defense Fund, is threatening to take FDA to court

to halt licensing of the test until "adequate safeguards" are adopted to prevent misuse.

FDA last week took some hasty action to allay concern by calling a meeting at which the five manufacturers developing the tests "voluntarily" waived their proprietary rights to confidentiality and announced the results of clinical trials to their products. Abbott Laboratories, which is considered to have an edge over its competitors — certainly in production capacity — announced that its test accurately detected 95 per cent of a group of 119 diagnosed AIDS patients; when tested on 17,000 random blood donors, 99.8 per cent came up negative. That rate is within the range that might plausibly be expected for the general public.

FDA also announced that it is sending out a letter to 500,000 physicians in the United States warning that the test's primary purpose is screening the blood supply and that it is not a test for AIDS but only for antibody to AIDS, which may mean any number of things; that the patient will develop AIDS in a few years, that he is asymptotically carrying the virus but will never become ill; and that the test has significant limitations when applied outside a research setting to asymptomatic persons — where the false-positive problem most noticeably manifests itself.

Once the test is licensed, however, FDA is powerless to limit its use by physicians.

Meanwhile, the statistics on AIDS incidence continue to mount. CDC now list 8,500 reported cases of AIDS; given the delays in reporting, CDC estimate that as many as 1,700 additional cases may already have been diagnosed and will be reported within the next three months. The number of cases is expected to double by the end of the year.

Stephen Budiansky

Animal welfare

Protesters as laboratory animals

ANIMAL rights groups in Britain seem to be making modest headway. Last week, Bromley magistrates' court fined the Royal College of Surgeons £250 for causing unnecessary suffering to a macaque monkey at the college's Buckston Browne Research Establishment. This test case was brought by the British Union for the Abolition of Vivisection as the direct result of a raid on the research farm last summer. The monkey in question was severely dehydrated because of inadequate ventilation and high temperatures (85–92°F). The college plans to appeal.

Meanwhile, members of the Middlesex animal rights group have thrown down a challenge for researchers by offering themselves as subjects in anti-viral antibody testing, usually done with rabbits. The offer was made after the group was invited to tour the Colindale Public Health Laboratory last year, when the laboratory was opened to the public in an attempt to defuse local panic about work related to

Lassa fever. The laboratory has refused to perform the antibody testing experiments on human subjects because they would be unethical, unsafe, impractical and of limited scientific use.

Mr Peter Georgiades, representing the animal rights group, wants to discuss the offer further with the laboratory and to investigate other projects that could involve human volunteers. In the face of Colindale's refusal, the group plans to inform the Minister of Health of the offer.

Animal rights groups are unconvinced about government legislation on animal experiments. A Home Office white paper (policy document), supplementary to that produced in 1983 (see *Nature*, 303, 191; 1983), will be published "before Easter". It will propose stricter guidelines on the infliction of pain and suffering, but in Mr Georgiades' view, will not go far enough. Non-scientists should have more control over the experiments that scientists may perform, he believes.

Maxine Clarke

French education

Broadening the base for PhDs

ARE French universities in danger of becoming second-rate? Is French socialism driving an even deeper wedge between the universities and the elite *grandes écoles*, so increasing social divisions? Those are the fears of many university staff in France, who dread among other proposals of the present government the suggestion that the *grandes écoles* should be able to grant PhDs.

The one thing that has given the universities an edge over the *grandes écoles*, and a degree of pride, is that the former are, by and large, the only places to do research. Although the *grandes écoles* (engineering schools which are a legacy of Napoleon) train the cream of the French administration and business communities, and although competition for places in them dominates French education from primary schools upwards, they have until recently been content to leave research mostly to the universities.

But the present administration has been encouraging more *grandes écoles* to carry out research, like the military *Ecole Polytechnique* which already does important research in mathematics and physics (and which has recently entered biology). And now consideration is being given to allowing them to grant the new PhD research degree, a right won only this academic year by the universities as a replacement for their unwieldy *troisième cycle* and *d'état* degrees.

If the *grandes écoles* are able to award PhDs, some university staff argue, they will cream off all the best research students, leaving the university system, which is open to all comers, badly depleted.

The government's arguments are, however, pragmatic and clear. The *grandes écoles* are so well established in French society that they cannot be destroyed. And while they are training the French elite, it is better that they should train them in research. The French administration considers that one of the principal weaknesses of French industry is a lack of research experience at higher management levels. But there is a tendency among the *grandes écoles* students to despise the universities.

"The number of people in industry with research degrees is ridiculously low", says Bernard Descompes, director of research at the ministry of education. "Only 5 per cent of graduates (*ingénieurs*) of *grandes écoles* try to get doctorates, mostly in universities." That is 500 people out of an output of some 12,000 *grandes écoles* graduates a year, and of these only 100 end up in industrial laboratories, Descompes estimates.

"The best solution would be for the two sectors to set up cooperative research and teaching", says Descompes, "but the two systems won't join." The *grandes écoles* and universities seem like oil and water.

Thus the possible solution arises, to let the *grandes écoles* themselves grant PhDs. *Grandes écoles* such as those at Grenoble, Nancy and Toulouse that already have tendencies in that direction would be developed as technical universities.

M. Jean-Pierre Chevènement, leader of the Ceres left-wing faction of the socialist party and currently minister of education, seems to have no objection to such a development. M. Chevènement has recently given

French a new term, "*élitisme républicain*". It means, essentially, "to each according to his or her ability", whatever a person's social class. Chevènement has no wish to remove the *grandes écoles*: his logic is to strengthen them, but to remove social barriers to entry. But the *grandes écoles* are small institutions (there are a million students in French universities, twenty times the number at *grandes écoles*). If the *grandes écoles* cream off the best one-twentieth of research at French universities, will French science die or will it be invigorated? The experiment may be interesting.

Robert Walgate

US scholarships

Scholarship diplomacy booms

Washington

DESPITE proposed cutbacks in financial support for domestic students, assistance for foreign nationals studying and training in the United States is to be sharply increased. The contrast is in part explained by the circumstances that, according to one count, 33 heads of state and 450 cabinet ministers worldwide have at some time studied in the United States. The long-term influence of this "scholarship diplomacy" is reckoned to be considerable. Scholarships for students from developing countries offered by the Agency for International Development (AID) will increase by 50 per cent next year, to 15,000, and the US Information Agency will have doubled funds for its Fulbright grants for visiting students and scholars over the past four years. And there is probably still more to come.

A resurgence of congressional interest in assistance programmes for foreign students seems to have halted a long period of decline. While Soviet bloc educational programmes (excluding Cuba) tripled between 1972 and 1982, US scholarships declined by over 50 per cent over the same period. The executive branch, which tried to make further cutbacks in foreign student assistance during President Reagan's first term, now seems persuaded of their value.

Announcing the expanded AID programme, the agency's administrator, Peter McPherson, noted that in 1982 there were seven Soviet bloc scholarships for every one in the United States. The new AID effort will therefore be directed selectively at politically sensitive regions, particularly at disadvantaged students in Central America. Eventually, it is hoped that AID and the Information Agency could bring 10,000 Central American students to the United States each year.

The expansion of the AID participation training programme has been achieved largely by slimming down the agency's administration. The programme supports students in both training and "academic" courses. In 1983, 45 per cent of recipients were classed as taking academic courses. Over 60 per cent of students come from Africa and the Middle East. The new

expansion will for the first time allow the agency to support students on undergraduate courses, as well as the traditional training and postgraduate courses. The main focus will remain on development, however. There are also plans for collaboration with industry and universities, some of which reduce tuition fees for deserving cases.

The US Information Agency's Fulbright programme has benefited from the 1982 Pell amendment, which required a doubling of the Fulbright budget over four years. This target is likely to be met. The number of visiting students and scholars has now



reached the 2,000 mark after sinking to fewer than 1,500 a few years ago. Another award scheme run by the information agency chiefly for mid-career professionals, the Humphrey Fellowship scheme, is also now being expanded.

The outlook for foreign students in the United States is not so rosy for the great majority who are paying their own way, however. The high value of the dollar is being blamed for a marked slowing of the annual growth in numbers of foreign students. Tuition fees and living expenses commonly exceed \$20,000 a year. There is concern in some quarters that US academic institutions could be severely affected if foreign graduate students, in particular, stop coming. In many institutions, more than half of postgraduate students are foreigners, and they constitute an essential talent pool for faculty recruitment.

Tim Beardsley

Ozone depletion

Europe takes a cheerful view

Brussels

BELGIAN scientists Guy Brasseur and A. de Rudder, of the Belgian Institute for Space Aeronomy, claim that the threat posed to the ozone layer by the release of chlorofluorocarbons (CFCs) from aerosols is "distant" and "that time can be allowed without risk for further scientific evaluation of theory". The result of their model calculation will strengthen European Community opposition to a proposed global ban on aerosols propelled by CFCs put forward by the United States at a meeting of the United Nations Environmental Programme on the ozone convention in Geneva at the end of January.

One distinctive feature of their calculations is that they have taken account of the probable growth of emission of gases other than CFCs that act as ozone buffers (0.5 per cent a year carbon dioxide, 1.0 per cent a year methane and 0.2 per cent nitrogen oxides). They have calculated the effect on ozone perturbation of constant CFC emissions, a global phase-out of CFCs in aerosols over four years with a 3 per cent growth rate in emissions from other uses until 1997 and the same scenario but with an indefinite 3 per cent growth rate.

According to their calculations, ozone depletion will occur only in the case that CFC emissions grow by 3 per cent a year after an aerosol ban, and would not happen before the year 2034. But the imposition of a production capacity ceiling or "cap", coupled with an attempt to limit stratospheric chlorine, would lead to an actual increase of total ozone. From this, the two scientists have concluded that there is no call for an immediate ban which, they say, would achieve little over and above the considerable protection conferred by a production capacity cap.

Their findings back up present Community measures which involve a production capacity cap up to 480,000 tonnes of CFCs decided in 1980, corresponding to one and a half times the production quantity at the time, and an agreement among member states to reduce the use of CFCs as propellants in aerosols by 30 per cent on 1976 levels, which came into force in 1981. According to the European Commission, the 36 per cent level was reached in 1984.

Ministers had also agreed to monitor other uses of CFCs, in foam plastics, refrigeration, air conditioning and solvents, for which codes of practice have just been published by the European Commission.

The Community's approach to the problem of limiting CFC emissions, which has the support of the European chemical industry, is not, however, shared by such countries as the United States, which says the Community's proposal does not reduce emissions in the near term and is "tantamount to postponing action". It does not limit imports of CFCs or products con-

taining them, while provisions to exempt developing countries, as CFCs become more and more essential to their needs, will, according to the United States, result in undesirable CFC emission levels. The United States also maintains that the Community's proposal will entail economic penalties once the production cap has been reached and the use of other products, for which no safe substitutes exist, will have to be limited. Among the other uses is air conditioning, which is far more common in the United States than elsewhere.

The US Environmental Protection Agency (EPA) reckons that strict limits on non-essential aerosols (which are estimated to account for 36 per cent of total CFC emissions) will delay the Community production cap by 13 years and will slow down emission. European scientists, on the other hand, have calculated that the positive effect of a ban on the ozone depletion rate as proposed by the United States would probably be not more than about 0.2 per cent a year. Last month a group of countries known as the Toronto Group led by the United States presented their draft protocol on limiting CFCs which they would like to see laid down in the ozone convention due to be signed in Vienna in March.

Despite a distinct downward swing in computer modelling estimates of ozone depletion — depletion was estimated at 15-20 per cent in 1975 and at 2-4 per cent in 1984 — EPA projects remain gloomy. They put the new estimates down to a better understanding of the reactions among atmospheric gases but maintain that the increase of such gases as methane (and carbon dioxide) which act against ozone destruction will not be capable of stemming ozone depletion due to the considerable growth in CFCs. Unless action is taken, EPA expects an increase of between 1.4 and 4.2 per cent in CFC-propelled aerosols by 2075. Because of the non-linear relationship between CFCs and ozone levels, this could mean, they say, a 26 per cent depletion of the ozone layer by that time.

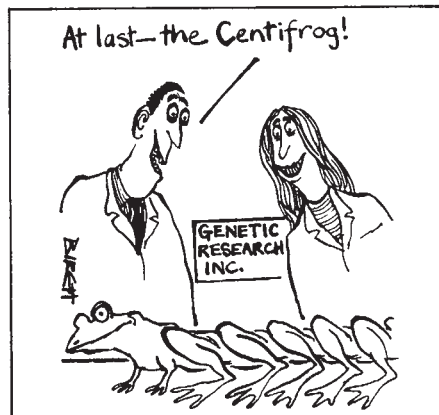
The proposal, drafted by the United States, which banned the use of CFCs in aerosols in 1978, concentrates on a major global reduction in the use of CFCs as aerosol propellants. It provides four options to achieve this: phasing down the use of CFCs by 80 per cent over six years, a ban on all non-essential CFC-propelled aerosols, a 20 per cent reduction in all CFC use and a production capacity cap as well as a 70 per cent reduction in aerosols. Any of these, it is claimed, could reduce cumulative emissions of CFCs by 7,600 million kilograms by the year 2010.

Anna Lubinska

European ban on frogs?

Brussels

MEMBERS of the European Parliament are increasing pressure on the Commission to ban imports of frogs' legs into the Community in an attempt to put an end to the alleged inhumane methods of slaughter and unhygienic conditions in which frogs are killed, as they become a more popular feature of the European dinner table.



Imports of frogs' legs into the Community have been rising steadily over the past five years. In 1983, according to the European Commission, 6,022 tonnes (the equivalent of 15 million frogs) was imported, of which 2,517 tonnes came from Indonesia, 1,577 tonnes from Bangladesh and 1,274 tonnes from India. The French are the principal consumers,

followed by the Benelux countries.

Apart from the inhumane way in which the frogs are killed, there is a risk of infection to consumers because of the delay between the killing and the packing in ice of the frogs' legs. Frogs are in any case natural pest controllers and in particular live off insects that may carry such diseases as malaria and encephalitis. It is also alleged that because so many frogs are killed for export to Europe, countries such as India are having to import chemical pesticides that would not otherwise be needed and which actually cost more than they receive for the butchered frogs.

Pesticides may also adversely affect frog spawning grounds, thus inhibiting population growth, as well as affecting the reproduction of birds of prey which normally live off rodents.

The conservationists believe that a ban on imports of frogs' legs into Europe would cut suppliers off from their markets and so destroy the incentive to hunt the frogs. Another possibility is that the edible frogs *Rana tigerina* and *Rana hexadecatyla* should be listed under the Convention on International Trade in Endangered Species, of which the Community is a signatory. But resistance from India, Bangladesh and Pakistan is so strong that this proposal may not even be tabled when the signatories to the convention meet in Buenos Aires in April.

Anna Lubinska

Human embryology

France seeks policy in haste

BRITAIN'S reaction to the ethical and legal questions surrounding *in vitro* fertilization, embryo research, the donorship of sperm and ova and surrogate motherhood has been a rush to legislate — with or without the advice of expert committees. In France, the approach, on the face of it, has been the opposite — to think first, to continue thinking and to leave the legislation until later.

But time is not on the French side. Quite apart from the rate of development and application of the science, France will be taking the lead in a debate later this year in the Council of Europe on legislative problems raised by the new techniques. So the French justice ministry is hastily trying to get its legal minds into some kind of order, leaving the deliberations of the French local and national ethical committees, which are attached to the ministry of health, to one side, offended by justice minister Robert Badinter's haste. The result is a muddle almost equal to that in Britain.

But the French political philosophy, at least, is clear enough. According to President François Mitterrand, "everything that touches life itself concerns each one of us, and nobody can decide *a priori* what is good or bad for his neighbour".

Thus, Mitterrand says, there should be no attempt to leave the judgements to "experts", as Britain has tried to do, perhaps unsuccessfully, with its committee on the issues chaired by a philosopher (Baroness Warnock). Rather, in France, Mitterrand wishes to see a wide public debate and public consensus before decisions are taken.

Indeed, Mitterrand even sees France, with its logical, philosophical and evolutionary history, as the place where these issues might first be democratically resolved. "In these domains", Mitterrand said at a conference held by Badinter last month, "France could play again the role it played at the end of the eighteenth century, when it was necessary to invent liberty and democracy."

All very well, but so far the French debate has been, in practice, heavily controlled, and experts have been well in evidence. The "national ethical committee", established by the medical research council INSERM (although now under the ministry of health) under the chairmanship of ex-president of the academy Professor Jean Bernard, held its first "public debate" on the issues last December. But Bernard is said to have dominated the meeting, and simply attempted to assure the public that all was well and that the scientists knew what they were doing.

Moreover, earlier last year, the committee had issued guidelines for embryo research that legal opinion now

considers useless. According to the committee, the use of embryos was legitimate if the ends were "therapeutic, diagnostic or scientific", but "the embryo must be recognized as a potential human being which is or has been living and which must be respected in everything".

These statements are contradictory, in the opinion of a leading professor of law, Michelle Gobert of the University of Paris II, who therefore considers that the national committee "has resolved nothing in the present state of French law". On other issues, French law is equally at sea. In the case of surrogate motherhood, for example, the legitimate father will be the husband of the surrogate mother, as French law generally assumes the husband of a pregnant woman to be the father, and he will have to disavow the child under Article 312 of the Civil Code if another man, such

as the genetic father, is to claim parenthood.

In such ways, then, French law is in no better state than that of other countries. And the debate, so far, is not much more advanced. This was perhaps why Badinter's colloquium on "Genetics, procreation and the law" last month brought together many experts in law, medicine, sociology, psychiatry and biology to present papers — Badinter and his colleagues wished to "bone up" on the issues. The setting of Badinter's meeting was, however, formal — and again there was little debate. Thus Mitterrand's ideal has yet to be realized in France.

The practical question now must be more mundane: will the ministry of health (to which Jean Bernard's national ethical committee reports) or Badinter's justice ministry (which must make the presentation to the Council of Europe) take the lead in what seems more and more likely to be just a hidden French version of "expert advice"?

Robert Walgate

Biotechnology

US speeds scrutiny and consent

Washington

AFTER a shaky start, the US Patent and Trademark Office is moving quickly to answer longstanding complaints of inconsistent and at times incompetent handling of biotechnology patent applications. The patent office plans almost to double its number of biotechnology patent examiners by the end of the year, with the aim of reducing the backlog of more than 2,600 pending applications and cutting the processing time from 28 to 18 months.

The patent office made a major change last year in the way it handles biotechnology patents when it consolidated its biotechnology examiners into a single section. Previously, a patent might go to the organic chemistry group if it was considered to deal mainly with nucleotides, to the polymer group if it was judged to be about peptides or to a third group if it was about fermentation products. These groups have now all been incorporated into group 120, which covers pharmaceuticals generally. Group 120 is now receiving virtually all applications involving genetic engineering and 90 per cent of the applications dealing with any aspect of what can be broadly defined as biotechnology. The number of biotechnology patent examiners will increase from 26 now to 40 by the end of the year. The number within that total dealing with genetic engineering will rise from 15 or so at present to 20.

Rene Tegtmeyer, assistant commissioner for patents, acknowledges the criticism that many of the older senior examiners who handled the first biotechnology applications did not know the technology. But Tegtmeyer claims that the problem is being solved by bringing in new blood, increasing in-house training programmes and

improving the patent office's information resources. These now include all commercial databases as well as Georgetown University's semi-private database.

Patent attorneys practising in the biotechnology areas agree that these are steps in the right direction. But one leading genetic engineering patent attorney notes that inconsistencies persist because of the gulf between the senior examiners, who are well versed in patent law and patent-office procedures but deficient in technical expertise, and the newly hired examiners who understand the science but not the law.

The inconsistencies have highlighted two major issues — the scope of and the disclosure requirements for patents. At the outset, the patent office was approving very broad patents in genetic engineering, of which the best known is the Cohen-Boyer patent covering the basic procedure for inserting foreign DNA into a bacterium using a plasmid vector. The patent office is now, patent attorneys say, going to the other extreme, and rejecting many broad claims. The attorneys say it will be some time before a proper balance is attained.

The issue of disclosure requirements has likewise swung to and fro. Some applications have been approved without the applicant having made a deposit of key starting material in a publicly accessible culture depository, such as the American Type Culture Collection. More recently, others have been rejected on these grounds. Several cases now pending before the board of patent appeals may settle this issue.

Despite the complaints, the US patent office appears to be well ahead of both Japan and Europe in dealing with the flood of new biotechnology applications.

Stephen Budiansky

Yugoslav science

Discontent in abundance

A SWINGING indictment of the implementation of Yugoslav science policy over the past few years emerged from a federal conference of the Socialist Alliance of Working People of Yugoslavia, held earlier this month in Belgrade. Although the persistent and worsening economic crisis can be blamed for some deficiencies, the conference was told that the main problem is that Yugoslav decision makers and society at large undervalue science, in spite of official pronouncements to the contrary.

Unless there is a radical change of this attitude, warned Academician Branislav Soksic, the leading Belgrade economist who opened the conference, the "exciting developments of world science and of the scientific-technological revolution" would bypass Yugoslavia. Science, he urged, must be funded not as *de facto* social expenditure but as a "paramount and decisive productive force".

Because of the economic crisis, Yugoslav science spending fell from 1.07 per cent of the national income in 1978 to 0.91 per cent in 1983. Yet limited resources are often wasted. Lack of coordination means that research projects and expensive equipment are duplicated. Moreover, the number of foreign licences and patents purchased each year continues to increase, in spite of the directives of the Third Congress of Self-Management in 1981, which called for the development of an indigenous base of science and technical know-how.

Yugoslavia has, numerically at least, a considerable scientific work-force. There are more than 830,000 engineers and technicians and some 26,000 researchers working in 850 institutes, 20 universities and eight academies of sciences (one for each constituent republic and autonomous province). According to Dr Branko Zezelj of the Federal Institute of Civil Engineering, the "knowledge and ability" of these people is grossly underrated, and they are provided with neither moral encouragement nor proper material incentives.

Other speakers at the conference, however, took a less rosy view, saying that because there is no "sound and constructive" criticism of scientists' work and qualifications, research support frequently goes not to those with "proven references" but to those who can "spin an attractive yarn".

Self-management procedures in appointing and reappointing the heads of research teams frequently become a cover for "negative selection" and the "fostering of mediocrity". Too often, therefore, competent scientists are driven into a triple brain-drain; from the underdeveloped areas into the cities, from science into more remunerative occupations and out of Yugoslavia altogether.

Even the most enthusiastic advocates of indigenous know-how would not suggest

that Yugoslav science and technology should become entirely self-sufficient. What they want is a more discriminating attitude to foreign achievements and products and the provision of "timely and selective" information about what is going on abroad.

On domestic research, failing increased funds, the conference decided that steps should be taken to deploy existing resources in as rational and careful a manner as possible. Duplication should be eliminated and there should be closer integration between higher education and the "independent" research institutions which enjoy the lion's share of resources. The conference also agreed that there should be a coordinated policy for science and technological development in each republic and autonomous province, while one speaker even called for a Federal Committee for Science, Technology and Information Science. In the most balkanized of all Balkan countries, this is brave talk indeed.

Vera Rich

French publishing

CNRS enters the market

THE major French research council, the Centre National de la Recherche Scientifique (CNRS), is to set up a publishing company "along the lines of Cambridge University Press", according to CNRS director Pierre Papon.

The company will begin with only a tiny capitalization (FF3 million, or £300,000) and a staff of 20, and will be based on an existing non-commercial (and loss-making) publishing administration within CNRS, Les Editions du CNRS. Papon hopes in the long run that the new company will make profits that could be fed back to benefit research, although the main objective is to improve French education in the sciences.

The existing Editions du CNRS selects texts only on academic merit, thus acting as a service to those CNRS researchers, mostly in the humanities, who publish their research work through books. Some 70 per cent of current CNRS titles are on humanities subjects. Occasionally, more by chance than design, the books are of general interest — such as a recent study of French foreign affairs and the Corps Diplomatique. This was well received by the Paris newspaper *Le Monde* and seems likely to reach a wide readership even when published at a price of some FF300 (£30) a volume, but such cases are rare.

Thus, according to Papon, CNRS, with a few exceptions, at present has "no editorial policy" — by which he means in effect no commercial policy. The new company, dubbed Les Presses du CNRS, will be different.

"CNRS has two publishing goals", says

Tunguska observed

DR P.W. Francis of the Open University has drawn attention to the following letter which appeared in *The Times* (London) on 3 July 1908, three days after the Tunguska event in Siberia at 7.14 a.m. on 30 June.

CURIOUS SUN EFFECTS AT NIGHT

TO THE EDITOR OF THE TIMES

Sir,— Struck with the unusual brightness of the heavens, the band of golfers staying here strolled towards the links at 11 o'clock last evening in order that they might obtain an uninterrupted view of the phenomenon. Looking northwards across the sea they found that the sky had the appearance of a dying sunset of exquisite beauty. This not only lasted but actually grew both in extent and intensity till 2.30 this morning, when driving clouds from the East obliterated the gorgeous colouring. I myself was aroused from sleep at 1.15, and so strong was the light at this hour that I could read a book by it in my chamber quite comfortably. At 1.45 the whole sky, N. and N.E., was a delicate salmon pink, and the birds began their matutinal song. No doubt others will have noticed this phenomenon, but as Brancaster holds an almost unique position in facing north to the sea, we who are staying here had the best possible view of it.

Yours faithfully,

HOLCOMBE INGLEBY.

Dormy House Club, Brancaster, July 1.

Papon. One is to publish "scientific tools" of very limited, specialist readership, as at present. That will continue. But "we think we should also have good books, not novels, but broader scientific and technical books like those published by Cambridge University Press, Massachusetts Institute of Technology Press and so on".

This second broader objective cannot be achieved with the present service structure of Les Editions and "we need a commercial company". Under its relatively recent reconstitution, CNRS is free to establish and profit from commercial affiliates. Hence the new publishing company.

The new titles will include both popular works and textbooks. France lacks publishers with an interest in engineering, materials science and even social sciences, says Papon. Students, for example, of thermodynamics, nuclear physics and statistical mechanics often have to use US or British textbooks.

On the face of it, the market for such texts in French is not very great, but a new Swiss technical publishing company based on the technical high school of Lausanne was making a profit on similar books in French within two or three years of its establishment. Papon has visited the company. "They're selling three, four, five thousand copies of each book" in subjects such as informatics and architecture. The potential French-speaking market, apart from France, includes a small part of Switzerland, Belgium, Quebec, French Africa and parts of Asia.

Robert Walgate

US nuclear power

Close friends are enemies

Washington

THE US Nuclear Regulatory Commission (NRC) has failed in its primary responsibility as protector of public safety and has instead behaved like a promoter of nuclear power, according to a document* put out by the Union of Concerned Scientists (UCS). It accuses NRC of deferring important safety issues by labelling them as "generic", of hampering public access to information and of failing to enforce even its own regulations.

UCS gives numerous examples where inadequate enforcement by NRC at an early stage of construction of a plant meant that changes had to be made later, often at enormous expense. The Zimmer Plant in Ohio, finally cancelled in 1984 after a painful series of investigations and accusations of improper conduct, is cited as an example of a plant killed by ineffective regulation. The Diablo Canyon plant, which is now operating, would have been in operation much sooner had there not been a breakdown in the quality assurance programme which caused some seismic protection modifications to be carried out with reversed blueprints. The list goes on.

The UCS report also reveals disturbing instances of apparent attempts within the commission to conceal reviews whose results could cast an unfavourable light on the commission or its senior staff. A federal grand jury is at present investigating allegations of criminal misconduct by some NRC officials. UCS also describes instances of NRC's unwillingness to co-operate in congressional investigations which have led to complaints by congressmen.

UCS says the delay in considering generic safety issues is leading directly to danger to public health. Several generic safety issues implicated in the Three-Mile Island accident, for example, have still not been resolved. NRC schedules are described as being "among the most flexible" in any branch of government. In other cases, safety regulations have been revised after it became clear that many plants would have to shut down temporarily in order to comply with regulations. UCS also notes that when NRC says a "generic" safety issue has been resolved, it means only that agreement has been reached on how the problem might be tackled.

UCS has a long history of involvement with nuclear safety issues and claims to have been instrumental in bringing to light safety issues that have later been accepted and enforced by NRC. The union's general position is that it believes nuclear power can be safely regulated but that NRC has so far failed to do this. Despite some evidence of a tightening-up of procedures at NRC after the 1979 Three-Mile Island accident, the

change was short-lived, according to UCS. The problem is not the competence of the NRC staff, but the attitudes of senior management, who set the tone for NRC conduct: "Like bureaucrats everywhere, the staff do what they think they are expected to do", in the words of John Pollard, a former NRC engineer who defected to UCS in 1976. The senior management, many of whom have been with the commission since it was formed as a spin-off from the Atomic Energy Commission in 1975, are accused of having too close a relationship with the industry.

The chairman of NRC, Nunzio Palladino, has said that while NRC welcomes constructive criticism, UCS "goes overboard". He pointed out that although UCS accuses it of putting the interests of the power industry first, the industry accuses it of being adversarial, so "we must be doing something right".

UCS proposes that NRC should establish deadlines for resolution of safety issues, and that a new Inspector General should be answerable to the President and Congress on NRC affairs. An independent Nuclear Safety Board is also proposed.

Despite optimistic assessments of the

future potential of nuclear power in the United States from industry groups, the fact remains that no new plants have been ordered since 1978 and that all plants ordered since 1974 on which construction had started have been cancelled. Both industry and NRC recognize that the licensing process has hampered construction and are pressing for reform; in particular, NRC recently proposed that it should be able to issue a combined construction permit and operating licence for standardized plant designs rather than hold separate inquiries, as at present. (The UCS report notes that holding an operating licence hearing when a billion-dollar plant has already been built almost guarantees the outcome will be approval.) The UCS critique agrees that a single decision on a proposed plant would be preferable to the present system, but insists that the design and the site location should be finalized before the inquiry is held. According to proposed legislation recently sent to Congress by NRC, however, the commission would be able to approve "essentially complete" designs and to grant site permits even if no specific application to build at a particular location had been received. But that, according to UCS, is a recipe for a continuation of the delays and expensive alterations that have plagued the industry so far.

Tim Beardsley

UK research councils

Straw in the wind

THE first resignation from the Natural Environment Research Council in the wake of publication of its corporate plan (*Nature* 14 February, p.517) has come about. Professor J.F. Dewey, a geologist from the University of Durham, last week announced that he had resigned and further embarrassed the council by saying why. The council had no comment to make earlier this week.

Dewey's resignation statement says that the publication of the corporate plan "brings to a head" long-standing problems about the council's role as the sole source of support for the earth sciences in Britain. He says there is a general consensus that economically important earth sciences are being neglected financially, and that the trouble is not so much the total budget allocation to the research council as the diversity of the council's responsibilities.

The statement goes on to complain of the plight of the British Geological Survey, which is said to be "enmeshed in an incongruous maze" of long-term strategic research "which is its proper function" and short-term contract research, where it has to compete with private organizations for contracts. Dewey complains that as a result, exploration for oil and gas in the North Sea has often to be carried out with the help of geological maps first made in Victorian times.

Dewey also complains about the pro-

posal in the council's corporate plan that the direction of research should in future be controlled by three senior members of the council based at its headquarters. Dewey says this is "a potentially very serious concept" that will generate more "showcase" research to the neglect of basic science.

In an unkind cut, Dewey says there is a case for transferring support for basic research in the earth sciences to the Science and Engineering Research Council. "To use the term environmental science" to describe their work suggests an inappropriate lack of rigour. "The essence of my view is that the council should no longer be responsible for the earth sciences."

Dewey's complaints closely echo others now common in the British earth sciences community, where the plight of the British Geological Survey is regarded as not much more serious than the place of geophysical techniques within classical geology. The postponement of British participation in the revamped International Deep-Sea Drilling programme at least until October is taken as a gloomy portent.

What can be done about the complaints remains to be seen. The Advisory Board for the Research Councils is too busy preparing financial applications for the year beginning in April 1986 to want to interfere at this stage, but ministers could change events by asking the right questions. □

* *Safety Second: A critical evaluation of the NRC's first decade.* Union of Concerned Scientists.

Japanese education

How to tamper with success?

Tokyo

SIX months into its discussions, Japan's Education Reform Council, set up to plan sweeping changes in the education system, is riven with internal strife. First recommendations to government are due in May or June, but the prospects of a national consensus seem to be receding.

Japan shares with the United States the feeling that something has gone deeply wrong with the educational system. But rather than indiscipline and falling standards, Japan is worried about overzealousness and excessive competition. On the surface, the two nations' educational systems are similar, after all the 6-3-3-4 system (the number of years spent at primary, junior, high school, senior high school and university respectively) was put in place by a team of US experts who spent four weeks in Japan at the end of the war. But differences in the two societies have completely altered the actual educational process.

Entrance to state universities, which are the most admired, cheapest and most sought after, can be caricatured as follows. In early spring, national examinations, which precede the examinations set by individual universities, are held for the 330,000 applicants for the 97,000 state university places. Seven subjects are tested in a one-thousand-point multiple-choice examination; emphasis is on rapid recall from a huge store of facts.

Surprisingly, results of this test are never announced. Instead, the student hurries from the examination hall clutching a copy of his marksheet and delivers it to a private examination company, the biggest of which will collect marksheets from two-thirds of all candidates. The company performs a computerized analysis, and puts students' marks together with accumulated data on the rankings of individual universities and their faculties. The result is an assessment of one's individual position in the hierarchy and the probability of one's being able to reach the entrance examination standard of any particular university.

Then comes the difficult bit: deciding which university entrance examination to try. There is only one chance each year because all state universities hold their examinations on the same day. Marks in the national examination will be added to those gained in the university examination (in a proportion that varies from university to university) to give the final score which alone determines success or failure.

Thanks to the computerized analysis, many students simply aim at the best college they now know they have a good chance of entering. The disadvantages are obvious: considerations of what a young person would really like to study become lost as students choose courses simply to match their marksheet scores, those who enter a particular course tend to be of such

similar ability that lecturers find them dull to teach and the many who aim too high have to spend another year cramming for a second try.

Driving this hellish system is the recognition that the rank of university one enters will to a large extent determine one's success in life. Entrants to the most influential universities are virtually assured of entering good jobs in top companies or government and university life itself is almost a holiday.

The effect of the examination hell is felt right back down the system. Not only do students spend hours after school at *juku* (cramming schools) but they also log up the highest homework hours in the world. These are added to long school hours which ensure that a Japanese high-school graduate has spent four more "years" at school than his US equivalent. And the examination system for senior high-school entrance has come to mirror that for universities. Before a choice of senior high school is made — and there will often be ten such schools in any school district of a large city — mock entrance examinations are taken at private examination schools. Analysis of these results once again defines one's position in the hierarchy and what school one may hope to enter.

Of course, the 70 per cent of students who do not go on to university might seem immune from all this struggling. But that is not actually the case; as high schools are unstreamed and orient themselves to entrance examinations, less talented students tend to be ignored — so much so that the 6-3-3 school system is often cynically referred to as the 7-5-3 system, meaning that only 70 per cent of primary-school children, 50 per cent of junior high-school and 30 per cent of senior high-school children can keep up with the course.

Reform is not surprisingly a popular issue and one that Prime Minister Yasuhiro Nakasone has made skilful use of. But so far the Education Reform Council Nakasone set up (see *Nature* 308, 677; 1984) seems bent on proving how difficult it is to achieve. The council is split into four committees: reform of education for the coming century, social education, elementary and junior high-school education and higher education. The first committee's brief is wide; it is this committee, calling for drastic change, that has clashed in recent weeks with the fourth committee, which is closer to the Ministry of Education, Science and Culture (MESC) and wants whatever is to be done to be done very slowly. Hottest debates have hinged on "liberalization", a word, it seems, with many meanings. At one level it implies what everyone agrees is needed, less emphasis on standardized scores and more on a student's individual needs. But for the first group, headed by Ministry of International Trade and Industry former vice-minister

Naohira Amaya, it also means "letting in the vitality of the private sector", massive widening of the regulations for setting up schools and colleges so that many more private schools can be set up, abolition of school catchment areas to allow "freer choice", making all high schools and universities into corporations so that the difference between state and private schools and universities vanishes, ending of centralized textbook selection by MESC and elimination of national examinations for entry into many professions.

Such strong opposition to these reforms has come from the fourth committee, along with a panel of former education ministers and the teachers' union (what those in favour of the reforms call "vested interests") that the word "liberalization" is to be dropped by the first committee; it is to be replaced by the less well-defined concept of "individualization" of education.

Three more concrete suggestions have, however, emerged to a better reception: that the university year should begin in September rather than April to facilitate exchange of students and staff with US and European colleges; establishment of one and only one common examination for all universities, public and private, with fewer subjects; and fusing junior and senior high schools to remove one stage of competition. The fear is, however, that these last two measures would simply change the form and timing of competition rather than reducing its ferocity.

The real problem is perhaps that in any society where academic achievement (or at least, university entrance) closely determines success later in life — and Japan is close to being an absolute meritocracy — there is certain to be relentless competition in school. But at the same time the clear knowledge that it is one's own measurable performance, and not one's social background or anything else, that determines one's fate has given Japan one of its greatest strengths: an absence of class consciousness. The clear understanding that everyone has a chance has done much to strengthen the unity of Japanese society. That feeling is further reinforced by an unwillingness to introduce school streaming, for that would mean rejecting some members of an established group.

Japan's education system, in producing high quality through competition, along with a sense of equality, may have done much to bring about Japan's economic miracle. It is hard to see any government greatly changing what has proved so successful, although the Education Council's report in a few months may contain some surprises.

If change comes, it may perhaps do so for other reasons: many companies are starting to feel that now Japan is an international economic power the products of the present education system are not quite what they want — a change in their recruiting procedures would have a very rapid effect.

Alun Anderson

Aftermath of nuclear war

SIR — The discussion in *Nature* over the past months (for example *Nature* 310, 621; 1983 and 312, 696; 1984) of the various models and calculations on the possibility of a nuclear winter is pertinent and valid. We should like to add two points.

First, it appears that for any one aspect of nuclear war aftermath being examined in detail — be it adequacy of civil defence, food storage or distribution, world economy, fate of political and social systems and so on — the possibility for complete collapse is present in every case, and its probability usually appears quite large.

Second, the overly detailed examination of minutiae of any one model for any one aspect may serve to deflect attention and concern from the primary, unavoidable and lasting result of a major nuclear war: the death and suffering of hundreds of millions of human beings, starting milliseconds after the first flash and continuing for years and generations.

PER OFTEDAL
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SIR — We should like to comment on the continuing debate over simulation of global effects of nuclear war¹. John Maddox's criticisms of the "nuclear winter" concept^{2,3} were later heartily endorsed by Sherwood Idso⁴. However, Idso's arguments deprecating simulation techniques are grossly misleading for readers not acquainted with simulation methodology. Objections of a similar nature — namely that it is easy to programme in what wants to get out and that simulation does not prove anything — are also often raised against other studies using simulation in non-technical fields. For this reason we wish to draw attention to the question of what a simulation result in these fields can actually do.

Analysis of this problem for simulation of biological systems⁵ has shown that possible outcomes of simulation are: (1) survey of missing knowledge, (2) refutation of a hypothesis on which the model was constructed, (3) quantified (calibrated) model representing a more accurate hypothesis, (4) prognosis estimating future development by means of the quantified model, (5) verified model having the character of scientific explanation and (6) scientific prediction by means of the verified model.

The underlying reasoning holds also for the simulation of global effects of nuclear war. In this case a complete model verification is inconvenient. The developed models of "nuclear winter" therefore cannot be used for accurate scientific prediction and applications are limited by the first

four types of outcome. A final proof demanded by Idso cannot be provided either by simulation or by any other means.

Further elaboration of models may bring about refutation, changes, refinement or further confirmation of the "nuclear winter" concept, but all this leads to enrichment of knowledge which is the aim of every scientific endeavour. The positive results of Turco *et al.* consist of testing plausibility of hypotheses and compatibility of assumptions and above all providing inspiration to further investigation, as has indeed been the case.

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Social Spencerism?

SIR — The first edition of *Nature* (I have your facsimile) was published on 4 November 1869, nine months before the Franco-Prussian war which led to the political unification of Germany under Bismarck and his Kaiser. A new powerful, dynamic Germany became a major historical force through two world wars until the dissolution of Bismarckian unity in 1945 after Hitler and National Socialism. A report of a meeting of German naturalists and physicians at Innsbruck in the Tyrol prepared for the first edition of *Nature* by Arch. Geikie states:

What specially struck me was the universal sway which the writings of Darwin now exercise over the German mind. You see it on every side, in private conversation, in printed papers, in all the many sections into which such a meeting as that at Innsbruck divides. Darwin's name is often mentioned, and always with the profoundest veneration. But even where no allusion is specially made to him, nay, even more markedly, where such allusion is absent, we see how thoroughly his doctrines have permeated the scientific mind even in those departments of knowledge, which might seem at first sight to be furthest from natural history. "You are still discussing in England", said a German friend to me, "whether or not the theory of Darwin can be true. We have got a long way beyond that here. His theory is now our common starting point."

Herbert Spencer, a political philosopher, sociologist and friend of Darwin, who is mentioned several times in *Origin of the Species*, the first edition of which was produced in 1859, persuaded Darwin to use the term "struggle for existence" in later

editions¹ and was to use the Darwinian ideas of "struggle for existence" and "survival of the fittest" in promoting his theories of *laissez-faire* capitalism which were popular especially in the United States of America until the turn of the century. Spencer was to use the authority of the great observer and thinker to bolster sociological concepts unrelated to the biological concepts of Darwin.

The confidence trick that Spencer perpetrated continues to exist in our history books where the Spencerian cuckoo is still sitting in the Darwinian nest within the arboreal history of ideas where it is known as Social Darwinism. When historians refer to "Social Darwinists" they mean militarists, nationalists, imperialists, racists or apologists for capitalism, who believe in a natural sociological pecking order which justified them morally in their use of social, economic and political power and force. It is my contention that historians are concealing and confusing issues by the use of the term "Social Darwinism" and if they must use a term of like nature they should preferably use "Social Spencerism"². Even better, let them think up some other all-embracing term since the term may be their own comparatively recent invention anyway¹.

Historians call Adolf Hitler a "Social Darwinist"³. Hitler was an anti-semitic, a racist, a militarist and dictator who was unconcerned about human death and suffering. In this he was like many other men and women in history but *they* are not called "Social Darwinists". Hitler and his irrationalism are the antithesis of Darwin and his rationalism and I object to historians taking Darwin's name in vain. A historian of ideas¹ has challenged those who believe that there was a scientific basis for National Socialism⁴ and that the popularization of Darwinism within Germany in the second half of the nineteenth century was partially to blame for the misdirection taken by the German nation.

The Reverend W. Tuckwell's article "Science Teaching in Schools" in the first edition of *Nature* reveals the state of education which preceded the Education Act passed by Gladstone in 1870 offering universal primary education in England. The nineteenth century may therefore be forgiven for not better understanding Darwin's ideas but now that they are understood, the pseudo-scientific and inherently anti-intellectual term, Social Darwinism, should be discarded.

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Uses for ancient eclipse records

In spite of arguments about the astronomical interpretation of ancient eclipse records, one thing is certain — the more that can be found, the more useful they will be.

THE sport of wringing information of value in astronomy from historical records has long since been made respectable. It is even tempting to wonder whether the present understanding of supernovae would have been possible without the Chinese record which was recognized, in retrospect, to be a first-hand account of the star from which the Crab nebula was formed (but with a pulsating neutron star left over). The reality of the Maunder sunspot minimum in the early seventeenth century was established (by J. Eddy) by poring over ancient records, this time, to be sure, contemporary astronomical records.

The use of ancient records of solar and lunar eclipses is even longer established. Robert R. Newton begins an elegant paper on the acceleration of the Earth's spin (*Geophys. J. R. astr. Soc.* **80**, 313-328; 1985) with an account of how Edmund Halley concluded from some observations of lunar eclipses due to Ptolemy that the length of the year had been decreasing. This implied, said Halley, "the necessity of the world's coming to an end, and consequently that it must have had a beginning, which hitherto has not been observed in anything that has been observed in Nature". For his part, Newton wonders how Halley could have come to the conclusion that the Sun was accelerating (when in reality, the opposite is the case) and asks a little wistfully that "if any reader knows the basis on which Halley found the Sun is accelerating, I would appreciate hearing of it".

Since much of Newton's own argument is concerned with demonstrating the pitfalls of using the records of eclipses, he should not be so surprised. The potential value of ancient eclipse data stems from the fact that they provide a nearly exact measurement of the relative longitude of the Sun and Moon (ideally zero for a solar eclipse and 180° for a lunar eclipse) at some distant epoch. In principle, the only changeable elements in this equation are the rate of the Earth's rotation on its axis and the angular velocity of the Moon, which are both affected by their mutual tidal interaction. In practice, so people have been arguing since Halley's time, it should then be possible to calculate from ancient eclipse observations the deceleration of the Earth's spin even as a function of time.

This is precisely what F.R. Stephenson and L.V. Morrison did at the Royal Society's meeting on rotation in the Solar System a year ago (*Phil. Trans. R. Soc. A* **313**, 47; 1984). Their objective, like

Newton's now, was to identify the secular change, whatever it may be, in the rate of the Earth's rotation. One obvious complication is that the calculated secular deceleration of the Earth's rotation attributable to tidal action is a mere 2.4 milliarc-seconds per century.

Stephenson and Morrison used a wealth of records, ancient and modern, spanning almost 2,700 years. The earliest data come from Babylonian records, both of solar eclipses and the Moon rising while already eclipsed, with a modest admixture of Chinese information. With the advent of telescopes (and accurate timekeeping) in the past three centuries, occultations of stars by the Moon have become a more accurate way of pinning down the data. One of the striking features of the data set is the poverty of the information available for the medieval period.

The mechanics of Stephenson and Morrison's analysis is outwardly simple. One neat way to describe it is by the difference between Universal Time (UT), astronomical time measured strictly by the Earth's rotation, and Ephemeris Time (ET), the smoothed version of UT introduced just over thirty years ago to provide a more uniform measure of the independent variable in the dynamics of the Solar System.

The transformation from one system to the other requires that allowance should be made for the acceleration of the Moon's longitude, supposed to be entirely the consequence of tidal interaction, which was originally taken to be -22.44 arc-seconds per century (and which Stephenson and Morrison think should be 26 in the same units, based on observations of the transit of Mercury). Then the difference between UT and ET at any stage should be a measure of the departures of the rate of the Earth's rotation from a fixed value.

The upshot of the Stephenson and Morrison analysis seems to be clear — for the past millennium, the secular change in the length of the day has amounted to 1.4 ms per century, but before that, the rate was greater, more like 2.4 ms per century. It goes without saying that, on the face of things, the more ancient records are in some ways the most telling — the difference between UT and ET increases with the square of the time elapsed.

And of course, the most ancient records do not depend on the timekeeping (if any) used for making the observations; provided that the date of observation is known (or can be calculated or inferred), the equa-

tions for the motion of the Sun and the Moon will suffice to fix the time at which an eclipse occurs so long as it is known where the event was seen (and so long as it can fairly be assumed to have been a total eclipse).

Newton's argument sets out to discard eclipse data that are for one reason or another unreliable. He spends more than a page of his printed paper demolishing the case for using as a datum the eclipse whose description is included in a poem by the Greek soldier-poet Archilochus, who is known to have divided his life between two islands in the Aegean. The date is what perplexes Newton, who concludes that the eclipse described was either that of 6 April, 647 BC or that of 15 April nine years later, and that a suitable choice of values for the acceleration of the Moon's longitude would have made it visible from either island. Both Dicke and Lyttleton, Newton says, used this eclipse in different connections.

Newton has some good clean fun at the expense of what he calls the "identification game" supposed to have been invented by Airey more than a century ago, in which people have been used to assuming a value for the lunar acceleration, using this to calculate past eclipses, using eclipse records to pick on one and using the result to recalculate the lunar acceleration. It is not surprising, Newton says, that the answer is usually not very different from the starting value, for the argument is circular. He is probably right to insist that such data do need careful scrutiny before they are used for serious purposes. The merit of his own analysis is its use of data such as those gathered by the Babylonians for the definition of their calendar consisting of measurements of the angular displacement between the Sun and the Moon at new moon.

Newton may have overlooked the way in which safety can be found in numbers, for within the uncertainties his conclusion is not sharply different from that of Stephenson and Morrison. Briefly, he concludes that the deceleration of the Earth's spin has declined by a factor of about two since 500 BC. He suggests that geomagnetism may provide the explanation. A host of others, such as post-glacial isostasy, would fit the bill. What stands out is that the ancient records, consistent among themselves, still have much more weight in estimating the secular deceleration of the spin than the more accurate modern measurements, befogged as they are by the irregular variations.

John Maddox

Microbiology

Crossroads for archaebacteria

from D.P. Kelly

Two extraordinary new microorganisms are described as independent reports in this issue of *Nature*. They are extraordinary not only for living in hot acidic mud holes but also, and more importantly, for seeming to represent a crossroad in the evolution of archaebacteria — an ancient line of bacteria which is distinct from other bacteria (eubacteria) and eukaryotes.

Karl Stetter and his colleagues have obtained their archaebacterium from solfataric craters in Italy. As reported on page 787, it grows best at pH 2 and at 90°C, but its outstanding claim on scientific attention is its ability to grow either as an aerobic autotroph, oxidizing sulphur with the production of sulphuric acid, or as an anaerobe, by the reduction of sulphur with hydrogen to hydrogen sulphide at very low redox potentials. Such metabolic versatility, alternatively oxidizing or reducing sulphur, has never before been reported or even thought to exist.

Many biologists are familiar with the activities of sulphate reducing bacteria (like *Desulfovibrio*) and sulphur-oxidizing bacteria (like *Thiobacillus*), the respective activities of which sustain a large part of the anaerobic and aerobic phases of the global sulphur cycle. While some enzymes and reactions in the reduction and oxidation of sulphur in these two types of bacteria are similar, the organisms themselves are physiologically, ecologically and phylogenetically very distinct from each other. In recent years, two seemingly distinct groups of thermophilic archaebacteria have been isolated from very hot natural environments. These are the extremely thermoacidophilic Sulfolobales, which can synthesize their organic requirements from inorganic precursors, obtaining energy from the aerobic oxidation of elemental sulphur even at pH 1.0, and the Thermoproteales, which grow at higher pH under anaerobic conditions and obtain energy from the oxidation of hydrogen coupled to the reduction of elemental sulphur to hydrogen sulphide. Although it has been reported that the anaerobic oxidation of sulphur is coupled to molybdate reduction in *Sulfolobus brierleyi*, it has not been considered that there might be any physiological 'overlap' between the activities of the sulphur-oxidizing *Sulfolobus* and the sulphur-reducing *Thermoproteus* species.

The isolation by Stetter's group of an organism that readily switches between such extremely different biochemical and physicochemical conditions for growth has many implications in biochemistry and phylogeny. By various means Stetter and his colleagues have proved that the switch is a phenotypic adaptation of the whole

population and not a result of multiplication of selected individuals within the cultures. By the criterion of DNA hybridization, the genomes of cultures growing anaerobically and aerobically are identical but are not significantly similar to the genomes of conceivably related bacteria, such as *Sulfolobus brierleyi*.

If one takes the view that the evolutionary sequence was from anaerobic sulphur-reducing energy conservation (as seen in present day Thermoproteales) to aerobic sulphur-oxidizing processes (as in the Sulfolobales) following the appearance of the oxygen atmosphere, then the new organisms represent a relict of the branch point in evolution from which the two extant groups arose. The metabolic versatility and complexity of the newly discovered organism is such that it cannot be regarded as a metabolically 'primitive' ancestor of the modern day Sulfolobales. Indeed, it

could well represent an evolutionary line from the Thermoproteales that is parallel to that of Sulfolobales.

The bacterium described by Wolfram Zillig and his colleagues on page 789 comes from a solfatar in Iceland which also yielded *Thermoproteus* in the enrichment cultures. It is similar to Stetter's, growing best at pH 2.5 and 85°C. The most exciting observation so far from this strain is that although its DNA-dependent RNA polymerases are identical whether it is growing aerobically or anaerobically, there are electrophoretic differences in the cellular proteins and a plasmid of 7,700 base pairs seems to be amplified five-fold during anaerobic sulphur-reducing growth.

The discovery of these remarkably versatile archaebacteria may thus have provided material not only for comparative biochemistry and phylogeny, but also for the development of a cloning vector to study information transfer and metabolic regulation in the Sulfolobales. □

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Macroevolution

The Red Queen put to the test

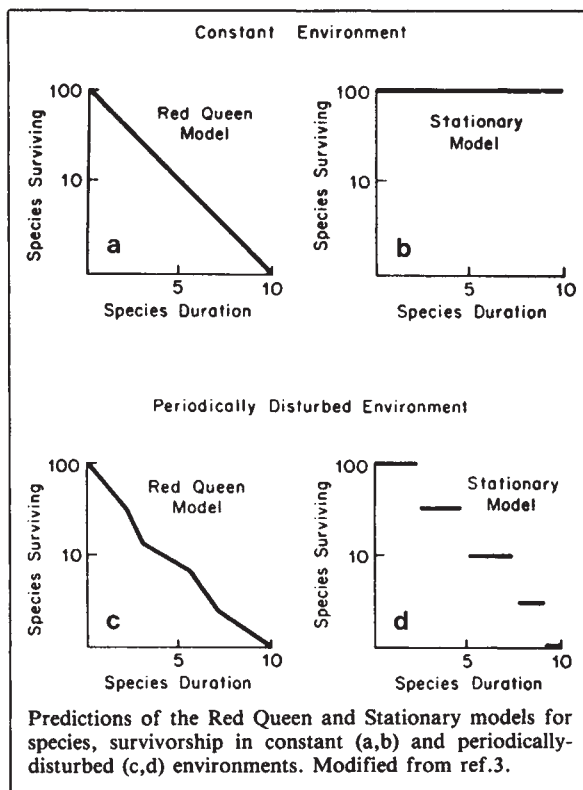
from Michael J. Benton

VAN Valen's Red Queen hypothesis¹ has attracted a great deal of interest since it was first proposed, and it has also been the subject of some controversy. Hitherto, discussions of the validity of the Red Queen have often been rather inconclusive because of the lack of an adequate test of the hypothesis. Now, Stenseth and Maynard Smith² have proposed a test, and two groups have already attempted to apply it^{3,4}.

The Red Queen hypothesis was based on the observation that, within any particular group of organisms, the probability of the extinction of any species or other taxon is constant through time (the Law of Constant Extinction). Thus, a species might disappear at any time, irrespective of how long that species has existed. Van Valen's explanation for this observation is that the various species within a community maintain constant relationships relative to each other, but that these interactions are constantly evolving. Thus, the antelope evolves greater speed in order to escape from the lion, but the lion evolves greater speed in order to catch its dinner, and so the status quo is maintained. Or, as the Red Queen put it in Lewis Carroll's *Through the Looking Glass*, "Now here, you see, it takes all the running you can do, to keep in the same place". Thus, species evolve continuously as a result of biotic interactions, and changes in the physical environment are not needed in order to propel evolution.

Several authors have questioned whether the Red Queen hypothesis is the only explanation of the Law of Constant Extinctions^{5,6}. There certainly seems to be a paradox if we consider that organisms may be continuously evolving and adapting, yet this process does not improve their overall chances of survival: the probability of extinction is independent of the age of a species.

Stenseth and Maynard Smith² contrast the Red Queen model, in which evolution is driven principally by biotic interactions, with a Stationary model, in which evolution is driven mainly by abiotic factors (for example, climatic change, or topographic change). The Stationary model is probably in better accord with traditional assumptions about evolution, and it predicts that evolution will cease in the absence of changes in abiotic parameters. Translated into practical terms, the two models should give quite different patterns of species evolution, and Stenseth and Maynard Smith suggest that an examination of the fossil record might provide a resolution between the two. The Red Queen model predicts constant rates of speciation, extinction and phyletic evolution in ecosystems, even when they are at equilibrium diversity, whereas the Stationary model predicts zero rates of evolution at equilibrium, interrupted by bursts of evolution, extinction and speciation in response to changes in the physical environment (see top pair of figures).



The main problem in applying the palaeontological test is to find an example in which no environmental change has occurred. Because such an example would be hard to demonstrate (if, indeed, one exists), Hoffman and Kitchell³ have allowed for the effects of a periodically disturbed environment in their application of the test by modifying the simple predicted patterns (see bottom pair of figures). The patterns are still distinctive: the Red Queen model gives an approximately straight line, while the Stationary model gives a distinctly stepped pattern. Their test uses data from detailed sampling of microfossils (coccoliths, foraminifera, radiolaria, diatoms, and others) from 111 deep-sea boreholes that sample the past 50 million years of sediments on the Pacific sea floor. The simple speciation and extinction curves obtained from these data are more or less smooth, rather than stepped, and so seem to support the Red Queen. An analysis of the cumulative appearance of new species also gives general support to the Red Queen although there is some evidence of stepping.

Further analysis shows there to be considerable variation in the probability of extinction over geological time: for example, there seem to have been particular periods in which all the microfossil groups had high extinction rates. These indicate plankton extinction events which would normally be attributed to sharp changes in the environment. When Hoffman and Kitchell make allowance for these events, the various analyses again point to the validity of the Red Queen model. On the other hand, Wei and Kennett⁴, who have also used the planktonic record as a test, regard their own results as weak evidence

for the Stationary model. Over the past 22 Myr, the diversity of planktonic organisms has changed sharply at times that correspond to environmental changes.

There are, however, several problems in applying these kinds of test. One problem concerns the difficulty of separating biotic from abiotic factors in order to assess their relative significance: it is probably impossible to pigeonhole both kinds of phenomena as independent factors. Second, in many real situations the test would be inconclusive. For example, it would be hard to distinguish the two predicted patterns if both curves were stepped as a result of rapid changes in the physical environment. At the other extreme, continuous small changes in environmental conditions might give similar gently sloping graphs for both models. Another problem concerns the assumption that species diversity reaches an equilibrium level, where speciation rate equals

extinction rate. The possibility of non-equilibrium models also has to be considered.

The debate between the two models should not be confused with that between gradualism and punctuated equilibrium. The Red Queen hypothesis is often seen as a gradualist interpretation of evolution, but neither viewpoint implies the other. The question of whether evolution is driven mainly by biotic interactions or by the physical environment is of great importance to all evolutionists and we may hope to see a great deal of palaeontological work in the next few years that addresses this problem. It is important to know if the Red Queen or the Stationary model has generality in all kinds of situations, or whether one model prevails in particular groups or in particular kinds of environments. Let us hope that palaeontological data are better suited to testing these two models than they have been for distinguishing gradualism from punctuated equilibrium. □

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Planetary science

Volcanic sulphur flows on Io

from Michael H. Carr

ON PAGE 778 of this issue, J.A. Naranjo describes the flow of molten sulphur at Lastarria volcano in Chile¹ and sulphur flow on the flanks of Mauna Loa in Hawaii has also recently been described². Although it is common to find sulphur in volcanic fumes, sulphur flows are rare on Earth and are of special interest for understanding volcanic activity on Io, innermost of Jupiter's four large satellites. Sulphur seems to play a prominent role in the intense volcanic activity of Io, but precisely how prominent is the subject of current debate.

Io is by far the most volcanically-active body in the Solar System. Its largest volcano, Loki, has been dissipating energy at a rate of over 10^{13} watts over the past few years³, equivalent to the Earth's total rate of loss of heat. Tidal energy drives Io's volcanism. Because of a resonance of its orbital motions with those of the other large jovian satellites, Io has an eccentric orbit, which results in changes in the sizes of the tides raised on its surface. The theory that this continuous flexure generates frictional heat, which could drive volcanism, was suggested just before the discovery of Io's volcanism during the Voyager encounters⁴. The original suggestion was that all

the tidal energy is dissipated within a thin elastic lithosphere, but it now seems that significant amounts of the energy are dissipated by visco-elastic deformation below the rigid lithosphere. Part of the generated heat is conducted outward through the crust but most is carried to the surface in molten rock.

A controversial issue is the relative role of sulphur and silicates in Io's volcanism. Shortly after the Voyager encounters, two models were put forward. In one, it was suggested that the surface is covered with a largely molten layer of sulphur, some kilometres thick, resting on a silicate sub-crust, which is visible only as occasional high mountains⁵. In this model, almost all the flows and volcanic constructs are composed of sulphur. In the alternative model, both silicate and sulphur volcanism occur, and the surface consists of interbedded sulphur and silicates⁶.

Why, despite recognizing the strong likelihood of some form of sulphur volcanism on Io, did the group who suggested the second model doubt that the satellite's surface is composed entirely of sulphur? After all, sulphur has been detected in the trail of neutral and ionized particles that Io leaves behind in its orbit; sulphur dioxide

has been positively identified in the infrared spectrum of the satellite's surface and a strong ultraviolet absorption could also be explained by sulphur; different allotropes of sulphur seem to match the different colours of Io's surface; black spots within its calderas have the same reflectivity as liquid sulphur; and temperatures of the hot spots on Io range up to 600 K, which is consistent with the presence of liquid sulphur. The main reason to doubt that sulphur alone constitutes Io's surface is scepticism that it has the strength to support the observed surface relief. Sulphur is an insulator and is ductile at lower temperatures than are silicates. At volcanic centres, where high conductive-heat flows are likely, temperature gradients in an all-sulphur crust would be steep, and sulphur should flow at relatively shallow depths. Therefore the presence on Io of calderas over 2 km deep suggests that the surface is not predominantly sulphur; the combination of thermal and strength properties required are more consistent with a surface rich in silicates.

Young has recently questioned the suggestion of abundant sulphur on Io on other grounds⁷. Shortly after the Voyager encounters, pictures of Io, richly coloured in tones of red, orange and yellow, were widely distributed. The different colours were generally attributed to different allotropes of sulphur. Precise calibration and examination of the spectral data has since shown that Io is yellowish-green, not orange-red, and that the supposed match between the colours of Io and different allotropes of sulphur is completely spurious. The only stable form of sulphur at Io's surface temperatures is S_8 (all other forms will revert to S_8 in hours) and so the absence of spectral evidence for other allotropes is not, in itself, fatal for the sulphur hypothesis. Young, however, maintains that discrepancies between the reflection spectra of Io and S_8 are so large as to rule out S_8 as a major surface component. He therefore questions the presence of sulphur flows on Io, and cites supporting morphological evidence. Pointing out the pitfalls of comparing spectra of pure elements with those of naturally occurring materials, which usually contain abundant impurities, others maintain that one cannot rule out the presence of sulphur in that way.

The most convincing evidence for sulphur volcanism on Io may be the surface temperatures. Three different kinds of hot spots have been recognized by the Voyager IRIS experiment: stable high-temperature sources of about 600 K; transient high-temperature sources of the same temperature; and stable low temperature sources of less than 400 K. All the sources are dark areas, mostly within calderas. Integrated infrared spectra of Io taken by telescope during eclipses have been successfully modelled with 10^{-5} – 10^{-6} of the surface close to 600 K, and none of it at higher temperatures. Even if only 10^{-8} – 10^{-9} of the surface is close to 1,000 K, it would be detectable in the spectra. The temperature measurements

are thus consistent with sulphur, which melts at 400 K, but not with silicates, which melt around 1,300 K.

Most investigators now believe that both silicate and sulphur volcanism occurs on Io. Injection of silicates into and on to the silicate/sulphur crust may remobilize the sulphur and cause flows, much in the manner described by Naranjo for Lastarria Volcano², but on a far larger scale. The mobilized sulphur could form pools within calderas where it may be maintained as a liquid by hot silicates beneath. Injection of silicates into the sulphur-rich crust may vaporize sulphur and sulphur dioxide to drive the volcanic plumes. Although studies of sulphur flows at terrestrial volcanoes are

valuable, resolution of the relative roles of sulphur and silicates on Io will have to await acquisition of high-resolution imaging and spectral data. It is planned that this information will be collected by the Galileo spacecraft, which is scheduled to fly by Io on 10 December 1988. □

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Protein structures

Two for the price of one

from Stephen C. Harrison

USING the techniques of molecular biology, large quantity of proteins can now be produced to order. One consequence is that it is becoming possible to determine the three-dimensional structures of new classes of proteins that were not previously available in sufficient quantities to crystallize. For example, on page 762 of this issue Ollis *et al.* report a crystallographic analysis of the large ('Klenow') fragment of Pol I, the DNA polymerase of *Escherichia coli* and the first polymerase structure to be solved¹. An accompanying paper on page 811, documents the similarity between the protein sequences of Pol I and the DNA polymerase of bacteriophage T7, and suggests an interesting three-dimensional relationship². This comparison neatly illustrates how knowledge of the three-dimensional structure of one protein can give useful information about the structure of its homologues and reminds us that recognizing similarity may be the only reliable way to predict structure from sequence.

The studies of Pol I by Arthur Kornberg and colleagues have a central role in the history of nucleic-acid enzymology, and it is satisfying to see initial three-dimensional correlates of this classic work. The Klenow fragment of Pol I is the carboxy-terminal two-thirds of the protein, which contains two of the three enzymatic activities (the polymerase and the editing exonuclease). As seen at 3.3 Å, it comprises two distinct domains — one of about 200 amino-acid residues, the other of about 400. The smaller domain, which roughly resembles the nucleotide-binding domain of kinases and dehydrogenases, binds deoxynucleotides; the position of the deoxynucleotide dTMP in the crystal probably marks the location of the editing exonuclease since deoxynucleotides inhibit this activity but not the polymerase.

The larger domain has a very deep cleft, about 20–24 Å wide and 30 Å deep. A six-

stranded β -sheet structure forms its base and several α -helical structures form its walls. The top of the cleft could be closed off by the folding down of a spatially-disordered subdomain (about 50 residues in the middle of the sequence, joining two α -helices that form one side of the cleft). Ollis *et al.* suggest that double-stranded DNA binds in this cleft, with the primer 3' terminus near the end that faces the smaller domain. In support, they cite the concentration of positively-charged amino-acid side chains in the cleft as well as the location of amino-acid changes in certain Pol I mutants.

The notion that the disordered subdomain might close down 'on top' of DNA bound in the cleft is an attractive way of explaining the ability of the enzyme to move along the DNA chain, and is reminiscent of proteins that bind to nucleic acids. Tyrosyl tRNA synthetase has a loosely attached domain, disordered in crystals, that might fold down on the tRNA³; the subunits of viruses such as tomato bushy stunt virus⁴ and southern bean mosaic virus⁵ have flexibility-tethered R domains to draw RNA into assembling shells; the tobacco mosaic virus subunit has a loop disordered in the protein disk that clamps around RNA in the virus⁶; the repressor of bacteriophage λ embraces DNA with N-terminal arms⁷; and the bacterial histone-like protein is thought to enfold DNA with a β -loop⁸.

Regarding the similarities between sequences of T7 DNA polymerase and the Klenow fragment of Pol I there are several striking points. First, the similarities occur in local segments of varying length (9–45 residues), eight of them in the larger domain. Second, these eight segments constitute most of the structures that form the cleft described above, including the flexibly-linked subdomain. Between these segments, the polypeptide chain in Pol I makes excursions to the surface of the do-

mains, and Ollis *et al.* believe that the insertions or deletions necessary to accommodate the T7 DNA polymerase could readily be formed².

Comparisons of the Pol I amino-acid sequence with those of various eukaryotic DNA polymerases do not reveal any significant alignments. As Ollis *et al.* point out, similar structures need not have obviously similar sequences (evolutionarily distant globins, for example), but the converse does generally seem to be true. Indeed, using a known structure to deduce the structure of a homologue is an approach that is as old as the first protein structures — haemoglobin structure was deduced from myoglobin, and trypsin from chymotrypsin. But now that nucleotide sequencing is leading to a rapid expansion in the library of amino-acid sequences, the detection of similarity takes on new importance.

It is clear that simple pair by pair comparisons of amino-acid sequences, while often revealing, have several limitations. Different criteria can lead to different alignments, especially in marginal cases. Even with the serine proteases, sequence alignment and three-dimensional alignment get out of register in the less conserved regions⁹. A more powerful approach is the detection of homology groups. A dramatic example is the analysis of sequences from bacterial repressor and activator proteins^{10,11}, where a conserved helix-turn-helix motif of three-dimensional structure can be predicted and a group alignment established, despite the fact that many individual pairs of sequences cannot be related unambiguously. Indeed, the alignment has permitted Wharton *et al.*¹² to predict the location of this motif, and hence the effects of certain *in vitro* mutagenesis experiments on two repressors whose structures have not yet been solved crystallographically. It should be noted *en passant* that a strictly comparable helix-turn-helix is not present in the large fragment of Pol I, although the J and K helices of the larger domain are reminiscent of the motif.

In other words, in predicting aspects of protein structure from sequence, what we need to recognize are entire domains or significant subdomains — not just elements of secondary structure. The poor correlation between local sequence and secondary structure has been aptly demonstrated by Kabsch and Sander¹³, who show that the same pentapeptide in different proteins has fundamentally different backbone configurations in each; before anyone runs a secondary-structure prediction program on a new sequence again they should carefully study this paper.

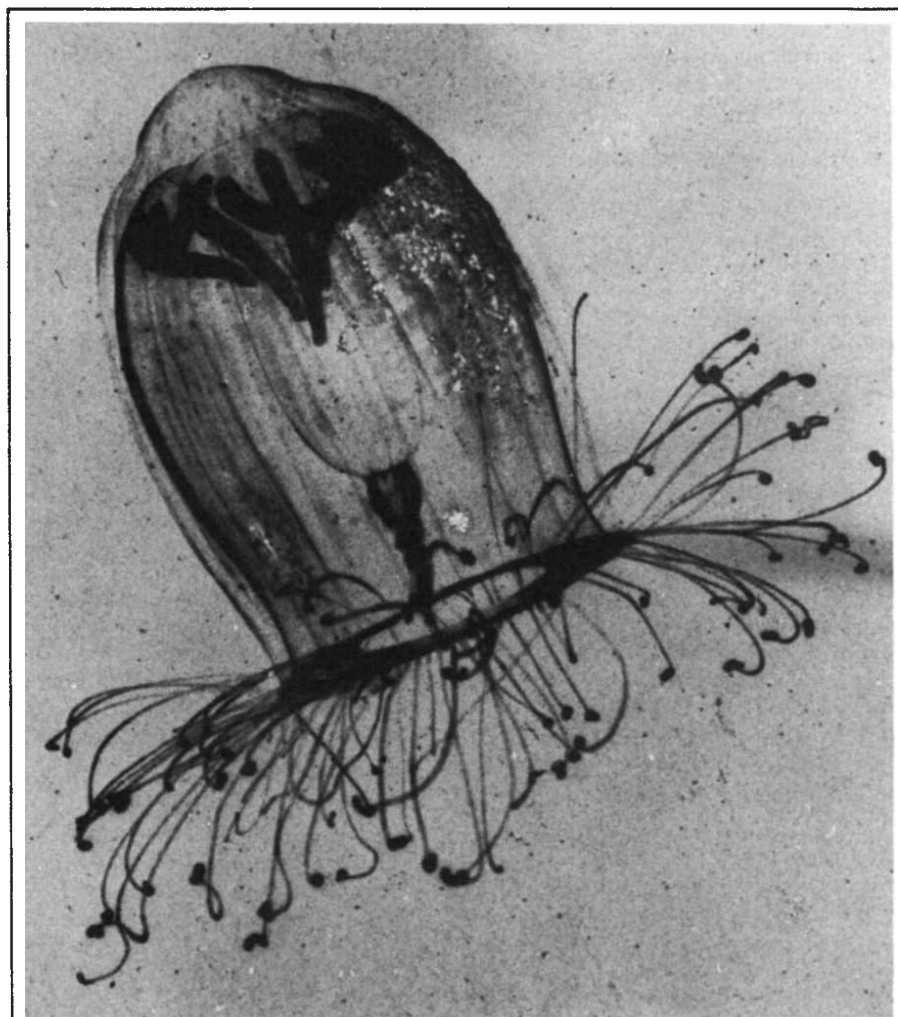
Thus, sequence similarity is currently the only reliable method for detecting structural similarity; several suggestions immediately follow. First, there is an important need for better ways to recognize similar sequences and to align them correctly. Will comparisons in more than two

dimensions (three or more sequences at once) be useful? Second, there is a real possibility that many interesting structures — or parts of structures — will be solved by 'catalogue look up', as used by Ollis *et al.* to suggest a structure for parts of T7 DNA polymerase.

Sequence data bases grow vaster each day. New crystal structures also appear more quickly (with the aid of two-dimensional position-sensitive detectors, as used by Ollis *et al.* and described in these columns last May¹⁴). If even some of the new structures tells us something about a whole family of homologues, progress will be rapid indeed. A solution to 'the protein folding problem' — given a primary sequence, divine the tertiary fold — has been regarded as the pot of gold at the end of the protein-crystallographer's rainbow. Perhaps a portion of the gold already lies at our feet. □

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Aglantha digitalis is a jellyfish that swims by contracting the wall of its bell-shaped body to expel a jet of water from its base. Swimming can be either slow, when it is fishing, or fast, when escaping from predators. One contraction during fast swimming takes the animal five times as far as in slow swimming, when it moves about 15 cm, one body length. In a paper on page 791 of this issue, G.O. Mackie and R.W. Meech show that both swimming behaviours are produced by the same muscle sheet, innervated by the same giant motor axons. Fast swimming is evoked by rapidly-conducted, sodium-dependent action potentials, whereas slow swimming results from a low-amplitude calcium potential in the same axon. This is the first time that two types of impulse propagation have been reported in a single nerve fibre.

Cell biology

Computer modelling of the behaviour of actin gels

from Dennis Bray

UNDERSTANDING certain kinds of cell movements may require the use of a computer, to judge from two recently published papers by theoretical biologists G.M. Odell¹ and G.F. Oster². This applies especially to the slow amoeboid movements of animal cells as they crawl over surfaces, engulf large particles or pinch into two in the course of cell division, movements which all depend on complex physico-chemical transformations in the amorphous protein gel just beneath the cell membrane.

While the biochemistry of the gel is fairly well understood (it contains actin, myosin and a medley of other proteins able to cross-link, fragment, cap or nucleate actin filaments—see ref. 3), its behaviour as a whole is difficult to predict. Most of the proteins are regulated by calcium ions, so that a transient wave of calcium through the cytoplasm is liable to be followed by solation, gelation, polymerisation and contraction. Moreover, the kinetics of these various effects are not the same, and transformations in one region of the gel have a mechanical and chemical influence on other regions. Seen from this perspective, even the simple extension of the leading edge of a fibroblast over a surface becomes a formidable skein of cause and effect.

Recognizing that only the language of mathematics is adequate to represent such interacting phenomena, Oster and Odell have been building detailed quantifiable models of actin gel behaviour. Their approach is to use a series of equations (also presented as word-pictures for non-mathematical readers) that represent the relationships between the stress, strain and viscosity of actin networks, and biochemical parameters such as calcium ion concentration. Combined into a multidimensional model, these provide an impressively accurate, and at times counter-intuitive, view of gel behaviour.

In the first paper that illustrates the genre, Odell presents a detailed descriptive model of the rapid repetitive shuttling of cytoplasm in the acellular slime mould *Physarum*¹. A series of biochemically plausible and experimentally verifiable equations describing the properties of an actin gel lead to the accompanying illustration of its behaviour. Here, the stretch imposed on a small volume of cytogel is related to the force it exerts and to the concentration of a diffusible regulatory component such as calcium. The fact that the resulting surface is saddle-shaped is crucial: there are two states in which the

gel exerts a large force separated by a region in which it is relatively weak. Consequently, if two separate regions of gel are linked mechanically, one will compete with the other and the combination will be inherently unstable. Built into a physical model of appropriate geometry, this property automatically leads to spontaneous rhythmic mechanochemical oscillations that have a close similarity to those seen in reality.

In a companion paper (which is mathematically less complete), Oster provides a number of valuable insights into the perplexing phenomenon of fibroblast locomotion². He points out, for example, that the fibres in any aqueous gel are under constant tension due to the osmotic tendency of water molecules to disperse them. Partial solation of one region of a gel therefore generates local osmotic pressure and hence local swelling. As a consequence, actin-fragmenting proteins, which solate actin gels in response to calcium signals, could in principle underlie the production of lamellipodia or microspikes from the fibroblast surface.

The potential power of these concepts is illustrated by the wide range of biological phenomena to which they can be applied. One of the earliest models, for example, describes the contractile properties of epithelial cell sheets⁴. In this case, the focus is on the apical actin filament arrays, which are assumed to undergo stretch-induced activation. Thus, when one cell contracts it will stretch other cells in the sheet, causing them to fire in turn, so propagating a wave of invagination. Computations on that basis produce a remarkably life-like simulation of sea urchin gastrulation, formation of the ventral furrow in *Drosophila* and neural tube formation in amphibia. Other models based on properties of actin gels have given rise to regular two-dimensional patterns of feather primordia and the skeletal rudiments in developing vertebrate limbs⁵.

Odell and Oster readily accept that their mathematical models are unlikely to be true. The equations correctly represent the observations, but the constraints are not too severe. After all, with two or more differential equations to conjure with, one has a hat that can produce any number of rabbits. But, at the least, the models demonstrate the rich potential for co-ordinated change in the mechanical properties of actin-rich gels. Moreover, they identify crucial questions of gel mechanics that now need to be, and probably can be, answered by experiment. □

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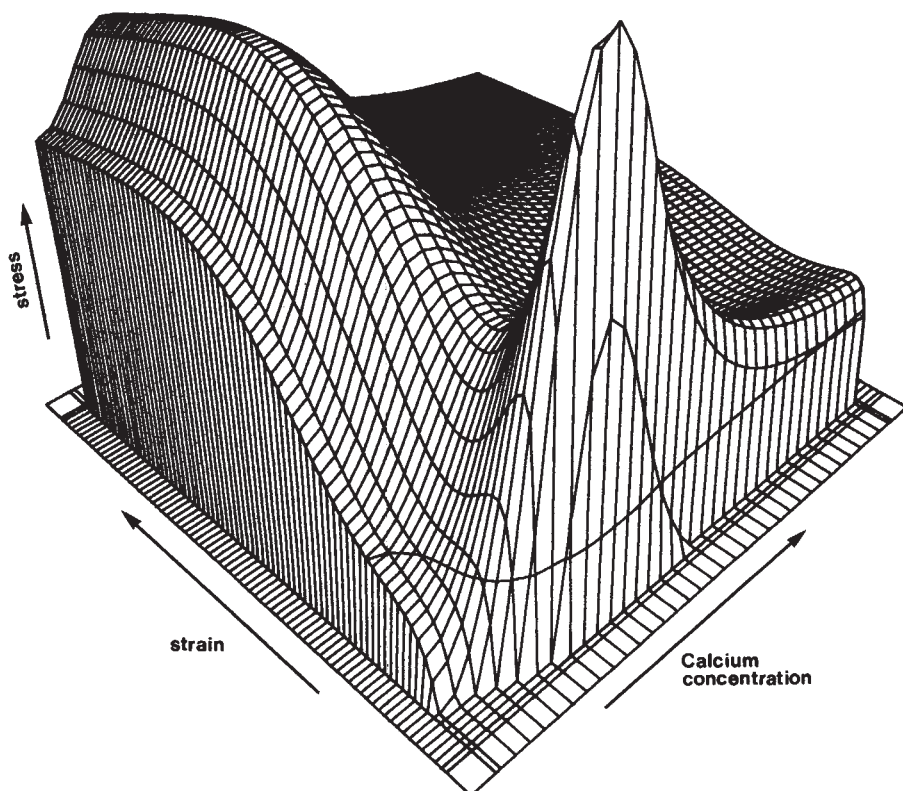
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Predicted mechanical properties of an actin-rich cytoplasmic gel. Modified from a paper of G.F. Oster in the *Journal of Embryology and Experimental Morphology* (ref. 2).

Palaeoecology

Polar forests of the past

from Simon Conway Morris

FOSSIL organisms or associations, in particular reefs and luxuriant vegetation, are regarded as amongst the most reliable indicators of past climates and environments. What, then, are we to make of the existence of polar forests in the Cretaceous and Lower Tertiary? The suggestion that they can be accounted for by a lower obliquity of the Earth then than now is impossible to maintain in the face of a paper published by E.J. Barron in *Geology*¹.

Cretaceous and Lower Tertiary fossil flora, include forests, where some trees have a trunk diameter greater than 50 cm at palaeolatitudes up to 85°, well beyond the present-day tree-line. Appeals to tectonic rafting from sunnier climes to a frigid docking may explain a few examples, such as those in Alaska², but the majority of forests appear to be genuinely *in situ*. Of these, probably the most remarkable is the fossil forest in Alexander Island, Antarctica for which Jefferson³ was able to provide a detailed reconstruction, including information on the original spacing of trees (about 1 per 17.5 m²) and flood-induced reaction wood. That these high-latitude environments must have been radically different from those existing today is self-evident. To explain the location of the polar forests, repeated appeals have been made to a marked decrease in the Earth's obliquity with sharply diminished seasonality and more evenly spaced photoperiods^{4,5}. Although palaeontology has played a not insignificant, if belated, role in upsetting other geophysical apocryphs, such as the fixity of continents, it has seemed increasingly improbable that shifts in obliquity during geological time⁶ are a realistic proposition. First, the celestial mechanisms to effect such a displacement are far from evident¹⁰, and now Barron's more earth-bound evidence looks like setting a firm seal on the debate.

Barron has modelled the likely temperature distributions on a Cretaceous world with the obliquity reduced from the present 23° to either 15° or 5°. His analysis relies on a general circulation atmospheric model coupled to a simple ocean model. Although making a number of simplifications, this seems to provide reliable first-order explanations. The paradoxical upshot is that a reduced obliquity exacerbates the problems of growing forests near the poles. This arises from a dramatic reduction in mean annual polar insolation, which more than off-sets the decreased seasonality and increased winter values of insolation, with a poleward transfer of heat that is unable to compensate for this loss. The calculated temperature distribution is not very different from that observed today (the northern Hemisphere would have been even

colder than now), so the existence of high-latitude forests is highly improbable.

In fact, the evidence for a warm and equable Cretaceous planet with no ice-caps or winter freezing is overwhelming^{1,11}, and it is the warmth at high latitudes that seems to resolve the enigma of their flora^{2,12,13}. Evidence suggests that temperature is more of a limiting factor than light for plants¹³ (for a dissenting view, see ref. 7), hence the flourishing tropical and subtropical flora of Swedish botanical gardens, where the glass-houses are heated and watered but receive only natural daylight². It is perhaps natural to think of Cretaceous forests engulfed in protracted polar nights but, contrary to the opinions of many authors (for example ref. 4), this is not incompatible with their existence. Today, annual insolation at locations well south of the Antarctic Circle can exceed that of East Anglia¹³ (which comes as no surprise to this writer). Moreover, the plants of high-latitude Cretaceous forests may have specific adaptations to intercept the maximum sunlight, including a conical shape and appropriate spacing of the trees¹³, large leaves¹⁴, or various physiological adaptations, especially amongst the evergreens, that enabled them to overwinter². Calculations suggest that not only would there have been a sufficient growing season with 360° illumination for two months of the summer, but the annual biomass laid down as woody tissue is well within the limits set by the available energy flux¹³.

The higher mean annual temperatures of the Cretaceous polar regions may be only a partial explanation for their biota. If levels of atmospheric CO₂ were higher than today¹⁵, this too may have had a beneficial influence on plant growth as well as helping to elevate planetary temperatures via the 'greenhouse effect' in the first place. Few authors¹³ appear to have given more than passing thought to this possibility, but the implications are intriguing. Concern about the release of anthropogenic CO₂ is leading to new assessments of the likely effects on plant life. Data are seriously wanting, but the bulk of evidence points to greater productivity and, perhaps, increased tolerance to stress factors such as extreme temperatures and drought¹⁶. Could it be that the Cretaceous and Lower Tertiary polar forests owe their existence in part to higher CO₂ levels? Experiments on living relatives of the fossil plants may well be illuminating in this respect.

Interactions between palaeoclimatologists and palaeontologists are of more than academic interest. Convincing climatic modelling, whether it be for the Recent or the Cretaceous, where palaeontological evidence provides independent tests,

strengthens belief that it is possible to predict accurately the effects of major increases in CO₂ or the reality of a nuclear winter (see *Nature News & Views* 312, 593; 1984). When the planet next reverts to a Cretaceous climate, it seems more than likely that the forests will return to the Antarctic peninsula, while alligators bask in the long polar summers and hibernate through the torpid winters. □

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100 years ago

ROYAL SOCIETY February 12,—"On the Underground Temperatures, with Observations on the Conductivity of Rocks, on the Thermal Effects of Saturation an Imbibition, and on a Special Source of Heat in Mountain Ranges." By Joseph Prestwich, M.A., F.R.S., Professor of Geology in the University of Oxford.

The author remarks on the difference of opinion between physicists and geologists respecting the probable thickness of the outer crust of the earth. The question of underground temperature, which is a subject equally affecting the argument on both sides, had engaged the author's attention in connection with an inquiry respecting volcanic action, and he was induced to tabulate the results to see how far the usually received rates of increase were affected by various interfering causes—not that most of them had not received due attention, but it was a question of whether sufficient allowance had been made for them.

Although Gensanne's first experiments were made in 1740, and others were subsequently made by Daubuisson, Saussure, and Cordier, in coal and other mines, it was not until the construction of deep artesian wells commenced in the second quarter of this century, and Walferdin introduced his overflow thermometer, and precautions were taken against pressure, that the more reliable observations were made. But notwithstanding the precautions taken, and the accuracy of the experiments, they present very wide differences in the thermometric gradient. From *Nature* 31 399, 26 February 1885.

Cell-surface proteins

At last the insulin receptor

from Tony Hunter

OF ALL the cell-surface receptors for peptide hormones, none can have had so much attention devoted to it over the years as the insulin receptor. Great effort has gone into its purification and structural characterization with the aim of determining how the insulin 'signal' is converted into metabolic events within the cell. Now, at last, as reported on page 756, the amino-acid sequence of the insulin receptor has been determined, not directly but from a DNA sequence¹. If there is a hint of disappointment about the result it is that there is no overall similarity between the sequence and that of any oncogene product, to parallel the identity of the *v-erb-B* oncogene product and part of the human epidermal growth factor (EGF) receptor². Nonetheless the sequence has some similarities to the products of a class of oncogenes and some unexpected features.

Previous work³ had shown that the insulin receptor has two distinct chains, α and β , which are derived from a single precursor. The receptor is usually found as a tetramer, in which two pairs of disulphide-bonded α - β dimers are joined by disulphide bonds between their α -chains. Because it can be experimentally cross-linked to insulin, the α -chain, which is glycosylated, probably forms the insulin-binding site. The β -chain, which is less glycosylated and contains phosphorylated serines, is phosphorylated on one or more tyrosines in response to insulin binding, both *in vitro* and in intact cells. Purified receptor can phosphorylate tyrosines of a variety of protein and peptide substrates in a reaction which is stimulated by insulin. This protein-tyrosine kinase activity, like that of the EGF receptor, seems intrinsic to the insulin receptor and is located on the β -chain.

To clone insulin receptor DNA, Ullrich *et al.* synthesized two long oligonucleotide probes on the basis of amino-terminal sequences of each chain and used them to screen a placental cDNA library. A single clone which hybridized with both probes was obtained; its nucleotide sequence indicates that it corresponds to an almost complete messenger RNA from the insulin receptor's gene.

The nucleotide sequence predicts a receptor protein of 1,370 amino acids. The start of the α -chain is recognizable 27 amino-acid residues down-stream from the predicted amino terminus of the protein. The intervening residues are largely hydrophobic, and Ullrich *et al.* suggest that they constitute a cleaved 'signal sequence'. The recognizable start of the β -chain is preceded by a four amino-acid sequence that is typical of processing signals for cleavage of precursors. Cleavage would generate a mature α -chain with 720 residues

and a β -chain with 620 residues. The α -chain does not have any stretches of uncharged amino-acids long enough to span a cell membrane, whereas there is a single, potentially membrane-spanning sequence in the β -chain. Therefore it seems likely that the whole of the α -chain is located outside the cell, together with 194 residues of the β -chain. There are fifteen recognizable glycosylation sites in the α -chain and four in the external portion of the β -chain, consistent with their relative degrees of glycosylation. The α -chain is rich in cysteines but the β -chain has only four cysteines in its external portion, at least one of which must be involved in a linkage to the α -chain, possibly the only (and indirect) attachment of the α -chain to the cell.

As anticipated, there is sequence similarity between the cytoplasmic portion of the β -chain, which contains the receptor's protein-tyrosine kinase activity, and the protein-tyrosine kinase domains of the products of the *src* gene family. All the established hallmarks of this catalytic domain are present in the β -chain sequence. As in the case of the EGF receptor and the product of the *fms* gene — a member of the *src* gene family — there are precisely 50 residues between the end of the transmembrane domain and the protein-tyrosine kinase domain. This region may contain the protease-sensitive site of the β -chain³. In the EGF receptor the equivalent linking sequence contains a threonine (Thr 654) that is phosphorylated by protein kinase C and that lies in the very basic sequence adjacent to the plasma membrane^{2,4}. Treatment of cells with tumour promoters, which are known to activate protein kinase C, causes *de novo* phosphorylation of this threonine, correlating with an inhibition of the usual EGF-induced increase in the protein-kinase activity of its receptor and a decrease in the receptor's affinity for EGF. In the case of the insulin receptor, tumour promoters cause an overall increase in the phosphorylation of its serine residues but no new phosphorylation sites are detectable^{5,6}. There is little change in affinity as a consequence of this phosphorylation, but a decrease in insulin-induced autophosphorylation of the receptor has been noted⁶. Although, as in the EGF receptor, basic amino acids are present following the transmembrane domain of the insulin receptor, this region does not contain a serine or threonine that could be a protein kinase C phosphorylation site equivalent to Thr 654 of the EGF receptor. Furthermore, purified insulin receptor does not seem to be a good substrate for protein kinase C. Therefore the effects of tumour promoters on the insulin receptor may not be mediated by

direct action of protein kinase C.

There are about 90 residues to the carboxy-terminal side of the protein-tyrosine kinase domain in the insulin receptor compared with 240 residues in the EGF receptor. In the latter, this domain contains the major autophosphorylation sites and is thought to serve a regulatory function⁴. Little is known about the location of the autophosphorylation site(s) in the β -chain of the insulin receptor, but Tyr 1316 and Tyr 1322 in its carboxy-terminal domain are possibilities, being in the vicinity of a pair of glutamic acid residues, which are common features of other tyrosine phosphorylation sites. In addition, there is a tyrosine (Tyr 1150) equivalent to the major site of autophosphorylation in the product of the viral *src* oncogene. Ullrich *et al.* suggest that Tyr 960 could also be a site of autophosphorylation. The identification of the site(s) is important because autophosphorylation of the receptor results in increased phosphotransferase activity⁷.

Given the identity of part of the EGF receptor gene to the *v-erb-B* oncogene², it is natural to ask whether the insulin receptor is closely related to a known oncogene product. Within the protein-tyrosine kinase domain, the receptor has partial homology with all nine members of the *src* gene family. Even taking into account the divergent species of origin, however, it seems that the insulin receptor gene is not identical to any of these genes. It will obviously be interesting to test whether or not an appropriate region of the insulin receptor gene is oncogenic. The best match of the protein-tyrosine kinase domain of the receptor is to that of the product of the *v-ros* oncogene of the UR2 avian sarcoma virus (52 per cent match of residues 990-1251 of the receptor to residues 264-529 of the *v-ros* protein). Outside this domain the *v-ros* protein and the insulin receptor do not appear to be related. Intriguingly, the predicted *v-ros* protein has a 28-residue 'transmembrane domain', located 68 amino acids to the amino-terminal side of the protein-tyrosine kinase domain. Moreover, there is a potential protein kinase C phosphorylation site six residues to the carboxy-terminal side of the postulated membrane domain. These structural analogies with other receptors suggest that the *c-ros* protein — the product of the cellular equivalent of the *v-ros* oncogene — is a surface receptor. Little is known about the *c-ros* gene, transcripts of which are most abundant in kidney tissue.

Comparison of the α -chain sequence with those of other proteins yields an unexpected homology with the external domain of the EGF receptor¹. The first 450 residues of the two proteins have about 27 per cent homology. The significance of this homology is unclear, particularly since these receptors bind different ligands, but could be related to some interaction with more ubiquitous extracellular recognition systems or even with intracellular trafficking machinery. If there are such functions

for the external domains of receptors it might help explain their unexpectedly large sizes. It will obviously be important to determine the location and extent of the ligand-binding sites in each case.

Ullrich *et al.* show that there is probably a single gene for the insulin receptor in the human genome¹. As expected, it is expressed in placenta and in cell lines that have insulin receptors. A number of different mRNAs seem to be expressed from the gene; since the insulin-like growth factor 1 (IGF-1) receptor is very similar in structure and enzymatic function to the insulin receptor, it is possible that some of the mRNAs come from the IGF-1 receptor. Given the homology between the two receptors we can anticipate that this matter will be resolved shortly by the cloning and sequencing of the IGF-1 receptor.

Last time I wrote in these columns, I expressed the hope that a comparison of the structures of the different receptor protein-tyrosine kinases would give us clues to the mechanism of transmembrane signalling⁴. Although there are several obvious analogies between the structures of the insulin and EGF receptors, unfortunately no startling mechanistic insight emerges. Both receptors cross the membrane once and have protein-tyrosine kinase domains in similar locations. The differences between the sequences linking these domains to the transmembrane domains in the two receptors suggests that no defined sequence in this region is required for signalling. It is interesting that the insulin-binding subunit is linked to the enzymatic subunit through disulphide bonds, while in the EGF receptor they are part of the same polypeptide chain. Subunit-subunit interaction, however, is a common feature of many allosterically-regulated proteins. The dimeric nature of the insulin receptor may be significant; Ullrich *et al.*¹ suggest that the insulin-binding site could be formed by the two α -chains, yet there is evidence that the disulphide bonds between the two α -chains are not required for insulin binding nor for the biological response⁹. Certainly it is easier to imagine how ligand binding could transmit a signal through a dimer structure, with its inherent rigidity, than through a monomer.

Clearly ideas on mechanisms of signal transmission can now be tested by direct molecular manipulation of the insulin receptor and interchange of its domains with those of the EGF receptor. Perhaps we will also learn why EGF and insulin trigger such different responses. □

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Astronomy

A Seyfert galaxy joins the jet set

from Beverley J. Wills

THE Seyfert galaxy NGC 4151, a nearby active galaxy, is back in the pages of *Nature* again. But the revelation by Ulrich *et al.* on page 747 is the most exciting so far. Thanks to the intrepid International Ultraviolet Explorer satellite, we now have the first optical evidence for high speed jets of material squirted from the nucleus of an active galaxy.

A popular model of the nucleus of a quasar, suggested by many observations and in line with current theoretical ideas, consists of a spinning black hole whose gravitational field provides the basic energy for the enormous flux of electromagnetic and relativistic particles observed by analysis of the optical and ultraviolet spectrum. A large fraction of the flux forms beams, moving at bulk relativistic speeds outward along the axis of rotation and probably coupled to, and fed by, a high-temperature ($2-4 \times 10^4$ K) accretion disk around the black hole. Direct evidence for these 'jets', extending for parsecs or megaparsecs, comes from radioastronomical observations, especially Very Long Baseline Interferometry, which reveals 'superluminal' expansion of knots away from an active core, aligned with long, narrow and often very straight jets connecting the core with outer, extended (kiloparsecs) fossils of jet activity. The frequently observed one-sidedness of the narrow jets is attributed to relativistic Doppler enhancement of the flux from the approaching beam, although this interpretation is less satisfactory at very large distances from the nucleus. Two-sided jets are more common in lower luminosity objects, perhaps indicating lower bulk velocities.

The central 'engine' is surrounded by dense (about 10^9 atoms cm^{-3}), fast-moving cloudlets or filaments which are photoionized by the strong ultraviolet continuum from the engine and give rise to strong broad emission lines, which are typically 2,000 - 20,000 km s^{-1} wide. Photoionization calculations suggest that these cloudlets extend about 0.1 - 100 parsecs from the central ionizing source, depending on its luminosity.

For the lower luminosity quasars, like that embedded in NGC 4151, this prediction has recently been confirmed by observations of changing broad emission line fluxes as the cloudlets respond to time variations in the central ionizing flux, after travelling at the speed of light across the broad-line region (BLR). The BLR lies within a much larger (up to kiloparsec size), low-density region, approximately $10^4 - 10^7$ atoms cm^{-3} , which is also photoionized, and gives rise to relatively narrow emission lines, a few hundred km s^{-1} wide. In near-

by active galaxies, such as NGC 4151, the outer parts of this narrow-line region (NLR) are spatially resolved. Because of the long time it takes for light to travel across the NLR, these emission lines should not respond to changes in ionizing continuum over time spans that we can study, as is borne out by observation. NGC 4151, although its radio luminosity is too low for it to be classified as a radio galaxy or radio quasar, has evidence of small-scale (200 parsecs) radio jets (Booler, R.V. *et al.* *Mon. Not. R. astr. soc.* **199**, 229; 1982) that seem to be related to, and interacting with, outflowing NLR material (Heckman, T.M. & Balick, B. *Astrophys. J.* **268**, 102; 1983).

Ulrich *et al.* now report two newly-detected narrow (less than 1,500 - 3,000 km s^{-1}) emission lines, straddling the broad C IV $\lambda 1549$ line in the spectrum of the nucleus of NGC 4151. These show up four-fold variations in strength over a few days and are apparently not identifiable in the rest frame of the galaxy. The authors suggest that these lines may be Doppler-shifted C IV $\lambda 1549$ arising from shock-excited material in a two-sided jet. If this is true, then we have the first direct measurement of the velocity of material in the line of sight associated with an extragalactic jet; the actual velocity is suggested to be about 0.1 c. This discovery opens up the exciting possibility of studying the mysterious region near the base of the jets, using optical emission-line observations of the jet or jet-entrained material.

Ulrich *et al.* argue that if these lines are indeed C IV, they are most unlikely to be from two photoionized, independent ('free-flying') clouds, either falling in, falling out or orbiting. The observed velocities have varied very little over three years, and the strengths of the two lines change almost simultaneously (within 10 days). The latter limit is based essentially on only one fairly well-observed, rapid increase among several smaller fluctuations, and it is obviously desirable to confirm this and improve the limit on the variation of the relative wavelengths.

Unfortunately, it is going to be difficult to investigate this phenomenon in other galaxies. NGC 4151 is a special case because it is nearby and active, and so is bright and therefore easy to observe, despite its relatively low intrinsic luminosity. Recently, both its variable nuclear continuum and, because of its small BLR, the broad C IV wings have been faint, and it is these conditions which have enabled the narrow line component to be discovered. □

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Digesting the Seascale data

SIR — The report of the Independent Advisory Group on the possible increased incidence of cancer in West Cumbria¹ has raised issues about the interpretation of occurrence rates for lymphoid malignancy, and of the associated cumulative Poisson probabilities, in the electoral wards of the Northern region.

M.J. Gardner (*Nature* 312, 16; 1984) rightly suggested that these two 'indices' bear upon different aspects of the data. The occurrence rate, which relates the recorded number of malignancies (for a particular ward over a defined period of time) to the size of the population at risk, may not be an accurate reflection of the 'true' or 'underlying' incidence rate because of the small size of the at-risk population involved in most of the wards. The recorded number of malignancies over any finite period of time is subject to an element of 'random' influence, taken account of in cumulative Poisson probabilities given in the Advisory Group report and by A. Pomiankowski (*Nature* 311, 100; 1984) which express the chance of getting the observed occurrence rate, or a higher one, if the underlying incidence rate for the ward in question is equal to the regional average rate of 0.61 per 1,000.

These Poisson probabilities, like the observed occurrence rates, must be interpreted in the light of the ward population size. The same observed occurrence rate of 5 per 1,000 in wards with at-risk populations of 200 and 400, for example, would give cumulative Poisson probabilities of 0.115 and 0.025 respectively. In other words, as Gardner pointed out, the Poisson probabilities are not a direct indicator of occurrence rate.

An alternative way of presenting the figures is by constructing a confidence interval for the underlying incidence rate. Confidence intervals at 95% and 99% confidence levels, for the wards with six highest observed rates of lymphoid malignancy, are shown in the table below. Although this alternative mode of presentation does not lend itself quite so readily to ranking as occurrence rates and Poisson probabilities, it does bring out two points with some clarity. First, the width of the intervals is a measure of how

relatively imprecise inferences made from these data are, because of the small ward sizes involved. The observed occurrence rate of 9.7 per 1,000 in the Seascale ward, for example, is consonant, at 95% confident level, with a true rate of anything between 2.65 and 24.9 per 1,000. Second though, despite this imprecision, the intervals give an indication of why the figures for Seascale are a cause for concern. Even at the higher confidence level of 99%, the minimum estimate of true incidence rate consonant with the data is 1.64 per 1,000, more than two-and-a-half times the regional average occurrence rate.

A related aspect of the small ward sizes, and the consequent imprecision of inferences made from the data, is the difficulty of detecting when the underlying incidence in a small area is raised above the regional average, even by substantial amounts. R. Wakeford (*Nature* 312, 191; 1984) expressed agreement with the Advisory Group conclusion that the Poisson probability calculated for the Seascale ward (0.000134), though extreme, does not justify the conclusion that Seascale is 'unique'. One must bear in mind, I think, that even if the true incidence rate in the Seascale ward were, say, *five times* the regional average, the chance of recording five or more occurrences (and thereby getting Poisson probability *more extreme* than 0.000134) would be less than 1%. The odds are clearly stacked against being able to reach any firm conclusions on occurrence data alone, even if a seriously inflated true incidence rate pertains.

Seascale was the only ward in western Cumbria picked out by Craft, Openshaw and Birch³ as having a Poisson probability less than 0.05. R. Wakeford suggested that this observation does not support the hypothesis of causal relationship between excess childhood lymphoid malignancies and increased radiation exposure around the Sellafield site. However, the small ward sizes, together with the 'discrete' nature of the Poisson probability distribution, imply that some caution must be exercised in interpreting a geographical pattern derived by reference to a specific cut-off value: in this case, the conventional 5% significance level. Two examples should suffice

to illustrate the point. Consider two hypothetical wards, one with a true incidence rate *three times* the regional average and an at-risk population size 100, the other with *twice* the regional average incidence rate and an at-risk population of 250. To get a Poisson probability of less than 0.05 in the first ward, 2 or more occurrences would need to be recorded (giving a Poisson probability of 0.0018, a good deal less than 0.05). The chance of this happening is less than 1.5%, even though the true incidence rate in this hypothetical ward is three times the regional average. In the second ward, again 2 or more occurrences would need to be recorded (giving a Poisson probability of 0.0105). The chance of this happening is less than 4%, despite the assumption of a true incidence rate double the regional average.

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Pinning down stem-cell populations

SIR — The proposal¹ that the only valid representatives of multipotent, 'persistent' haematopoietic stem cells are 'late' (≥ 12 day) spleen colonies has been critically discussed by Blackett². In support of such a critique, I quote a 1964 report³ showing that a number of 'early' (10-day) erythroid, unipotent and, according to ref. 1, 'transient' colonies do contain multipotent stem cells. In addition it has been shown that multipotent stem cells within many 'persistent', late (12-, 13-day) colonies do have *transient* potential for self renewal⁴. To avoid circular argument I propose that 'early' spleen colony counts adequately estimate the stem cell contents in a given sample. These counts are the most appropriate because they minimize the time-dependent colonial increase by stem cells migrating into test spleens from distant locations⁵. Obviously, the differentiative potentialities and renewal efficiency of stem cells within such colonies cannot be inferred from direct counts. These parameters have to be indirectly estimated by secondary transplantation procedures and interpreted exclusively in such contexts^{2,3,5}.

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West Cumbria lymphoid malignancy cases

Cases	Children	Observed rate per 1,000	Confidence intervals for true incidence rate	
			(95%)	(99%)
2	144	13.9	[1.68, 50.1]	[0.72, 64.4]
1	97	10.3	[0.26, 57.4]	[0.05, 76.6]
4*	411	9.7	[2.65, 24.9]	[1.64, 30.6]
1	165	6.1	[0.15, 33.8]	[0.03, 45.0]
1	172	5.8	[0.15, 32.4]	[0.03, 43.2]
1	174	5.7	[0.15, 32.0]	[0.03, 42.7]

Based on Table 40 of ref 2, showing 95% and 99% confidence levels.

* Seascale.

Relationship of AIDS to other retroviruses

SIR — Lymphadenopathy-associated virus (LAV)¹ is now considered to be the probable causative agent of acquired immune deficiency syndrome (AIDS). Other LAV-like viruses (the human T-cell leukaemia/lymphoma virus type III² and AIDS-associated retrovirus, ARV³) have been isolated and assumed, as some nomenclature would imply, to be a member of the HTLV superfamily of retrovirus which includes HTLV-I, HTLV-II and bovine leukaemia virus (BLV). Whether or not the AIDS virus indeed belongs to this 'superfamily' has become the subject of intense speculation.

The nucleotide sequences of both LAV⁴ and HTLV-III^{5,6}, as well as that of ARV⁷, allow definite comparisons with those of the HTLV/BLV group. First, the LAV and HTLV-III nucleotide sequences differ by less than 1% overall, whereas LAV and ARV differ by about 5% overall. The greatest sequence variation, both point mutations and insertion/deletions map to the *env* gene. Nevertheless, their deduced genetic organizations are identical, and differ from that of HTLV-I (Fig.1). Thus there is only a single AIDS retrovirus, and LAV, HTLV-III and ARV represent different isolates of the same virus. A hallmark of this virus is the presence, apart from the statutory *gag*, *pol* and *env* genes of a replication competent retrovirus, of two other open reading frames (ORF) which were called Q and F for LAV. The location of ORF Q is unprecedented, it overlaps with the end of *pol* and is followed by an apparently noncoding region, since hitherto the *pol* and *env* genes were contiguous. Directly 3' to *env* is ORF F which is half-encoded by the U3 element of the long terminal repeat.

We have also compared the deduced amino-acid sequences of LAV proteins with those of HTLV-I and other retroviruses and find no significant homology, except for domains *pol* and *gag* which are generally conserved among retroviruses. These regions probably correspond to the core structures of essential viral proteins.

Only the protein sequence of *pol* is conserved enough to allow computation of retroviral taxonomy. This we have done for the LAV *pol* sequence corresponding to the conserved regions of the endonuclease, as has already been reported elsewhere for a number of other retroviruses. The phylogeny of the LAV endonuclease se-

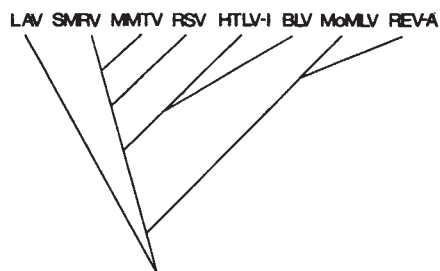


Fig.2 Phylogenetic tree of retroviral endonuclease amino-acid sequences. The tree was constructed using a partition analysis program, written by Drs William Saurin and Philippe Marliere at Institut Pasteur. The distances between branch points have no meaning. All subtrees generated by permutations of seven sequences were the same as that shown in the figure. In addition, trees constructed from segments of the eight sequences also produced identical trees. Our data are in perfect agreement with those of Sagata *et al.*¹¹ who, using an unweighted pair-group cluster method, produced the same tree as shown above for the SMRV, MMTV, RSV, HTLV-I, BLV and MoMLV sequences. The sequences corresponding to the conserved endonuclease sequence are, respectively, SMRV (squirrel monkey retrovirus 1-540, ref. 12), MMTV (mouse mammary tumour virus 1-540, ref. 12), RSV (Rous sarcoma virus 4,213-4,746, ref. 13), LAV (lymphadenopathy/AIDS virus 3,767-4,288, ref. 14), BLV (bovine leukaemia virus 3,982-4,530, ref. 11), MoMLV (Moloney-murine leukaemia virus 4,767-5,327, ref. 15) and REVA (reticuloendotheliosis virus A 4,969-9,526, ref. 16). The numbering of RSV, HTLV and MoMLV is as in EMBL data bank. Others correspond to those cited by the original authors.

quence (Fig.2) is that of a distant relative of all other endonuclease sequences so far established. The HTLV/BLV superfamily is most closely related to RSM, MMTV and SMRV subgroup (see Fig.2 legend). It is clear that at least the endonuclease domains of LAV and HTLV-I have been independently evolving for some considerable period of time. LAV cannot be amalgamated with the HTLV/BLV superfamily without taking a number of other retroviruses with it.

It is possible that LAV is in fact a member of the lentivirus family of retroviruses, as exemplified by visna virus⁸. We have previously noted their comparable genome sizes (9.2 kilobases including one LTR), and unusually large glycoproteins^{4,9}. Their base compositions are virtually identical (LAV: U = 22.2%, C = 17.8%, A = 35.8% and G = 24.2%; visna: U = 22%, C = 16%, A = 36% and G = 26%)^{4,10}. Using Southern blot-

ting under non-stringent conditions ($T_m = 55^\circ\text{C}$), we observed no DNA hybridization between cloned visna and LAV probes.

It is thus clear that the AIDS virus is not a member of the HTLV family of retroviruses and represents the prototype of a new class of human retrovirus, possibly a lentivirus. We propose to refer to the virus as lymphadenopathy/AIDS virus on the basis of the two main pathological effects of virus infection.

The absence of a close relationship between LAV and other retroviruses reinforces questions as to the origin of this virus and the cause of the recent emergence of AIDS.

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Microtubule assembly as a phase transition

SIR — In a *News and Views* item¹, J.R. McIntosh discusses two articles on microtubule assembly and disassembly². I would like to discuss a point of McIntosh's presentation where he refers to the famous condensation-polymerization model of Oosawa-Kasai (Oosawa later wrote a book on the same subject³): "... but unlike most phase transitions, the number of polymers formed increases rather slowly with increasing concentrations of subunits..."

If the effects of biological regulation such as GTP binding and hydrolysis are neglected, the autoassembly of biological fibres such as actin filaments, microtubules and microfilaments is one example of a general phenomenon called equilibrium polymerization where linear structures are formed in thermodynamic equilibrium. Other examples of equilibrium polymerization are found in several other areas of chemistry and physics: for instance, in liquid sulphur, long chains of sulphur atoms polymerize in equilibrium out of small S_8 rings^{4,5}; the viscosity of liquid sulphur rises suddenly around 167°C in a fully reversible manner. Certain ionic surfactants together with water and salt can

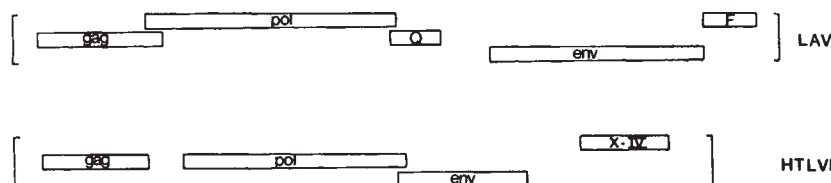


Fig.1 Organization of the viral genome of LAV and HTLV-I. Boxes represent open reading frames. Limits of the genome are indicated by brackets.

form very long cylindrical micelles⁶. Some organic polymers called 'living polymers' polymerize in equilibrium very suddenly^{8,9}. In the physics of adsorbed layers some phenomena such as commensurate-incommensurate transitions¹⁰ are driven by defect lines which behave like polymers in equilibrium.

Recently we have shown¹¹⁻¹³ that equilibrium polymerization can lead, if the initiation step is very difficult (very large free energy associated with the ends of the chains) to a quasi-second-order transition with a critical point fully analogous to the critical point of a ferromagnet in the presence of a very small magnetic field leading to a weak rounding of the transition. Thus, contrary to the statement of McIntosh, the condensation-polymerization model does present a phase transition very analogous to many other second-order phase transitions; in the limit of infinite free energy associated with the ends of the chains a true transition exists with the polymerization of very few infinitely long chains. Interestingly, the helix-coil transition¹⁴ is a trivial example of equilibrium polymerization in one dimension¹⁵ ($d = 1$) where helix portions are the 'polymers' and the 'coil' portions the unpolymerized 'monomers'. For this special low dimension case ($d = 1$) the transition becomes first order with a jump from all unpolymerized (coil) to all polymerized (helix). The effect of a non-infinite free energy of the extremities of the 'polymers' (frontier energy between helix and coil is not infinite) is to round the transition and to replace the first order jump by a sudden rise.

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a rather technical character which are not provoked by articles or letters previously published (where Matters Arising remains appropriate).

Periodic comet showers and planet X

SIR — In our recent *Nature* paper we discussed a model in which periodic comet showers are triggered by the passage of the perihelion of planet X through the edge of a trans-Neptune comet disk (*Nature* **313**, 36-38; 1985). It was assumed that all scattered comets with perihelia less than Neptune's orbit that ultimately became Jupiter family SP comets would contribute to the shower. However, it has recently been brought to our attention that the time scale for Neptune and Uranus captured comets to evolve into Jupiter family SP comets is greater than the interval between showers so these comets cannot contribute to a shower of duration of a few Myr (R. Muller and P. Weismann, personal communications).

This objection does not invalidate the basic model, since sharp showers will still be produced by comets scattered directly into Jupiter's sphere of influence, as Jupiter family SP comets have dynamical lifetimes ≤ 1 Myr. Direct scattering to Jupiter will require the inclination of the orbit of planet X to be $\geq 45^\circ$, which in turn will reduce the predicted semimajor axis of planet X from ~ 100 AU to ≤ 75 AU. The required large inclination is consistent with the failure of previous ecliptic surveys to find planet X. That fraction of Neptune-Uranus captured comets which ultimately end up as Jupiter family SP comets will contribute to (or perhaps dominate) the steady state background of SP comets. Preliminary estimates indicate that total numbers of shower and associated steady state SP comets are roughly comparable and that the requisite number of terrestrial impacts can be achieved.

A more extensive analysis of the planet X model taking into account the above modifications in the details of the shower mechanism, the effect of a thick comet disk on the shower time scale, and gap-diffusion driven by planet X, will be published elsewhere¹.

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The nomenclature of the heavy elements

SIR — Chemical elements, previously discovered in nature by chemists, in recent years have been artificially synthesized by nuclear physicists. Due to competition among chemists and controversy about the initial description of an element, some elements received two names, depending upon the country or chemist (for example beryllium or glucinium; niobium or columbium;

wolfram or tungsten).

The International Union of Pure and Applied Chemistry (IUPAC) has given its Commission on Nomenclature of Inorganic Chemistry the task of recommending a name for a newly discovered chemical element. However, Commission members are not experts in nuclear physics. Of late, there have been conflicting claims to discovery of the trans-lawrencium elements, $Z \geq 104$. IUPAC's policy, as written in the book of Nomenclature of Inorganic Chemistry, is not to deny the right of a discoverer to propose a name.

Among the international guidelines to consider are: the name should differ as little as possible in different languages and it should be based on pragmatism and prevailing usage. Since usage is a major factor, IUPAC's choice carries no implication about priority of discovery, for example some names ($Z < 92$) are not those originally proposed by the element's discoverer.

Meanwhile, theoreticians discuss the properties of undiscovered elements. Although elements do not receive common names before their discovery, names are needed for practicality, as well as for abstracting and retrieval purposes.

For these reasons only, the Nomenclature Commission proposed a system for naming any element with Z larger than 100 (*Pure appl. Chem.* **51**, 381; 1979). This is based upon the atomic number, with roots of 0 = nil, 1 = un, 2 = bi, 3 = tri, 4 = quad, 5 = pent, 6 = hex, 7 = sept, 8 = oct and 9 = enn. Thus, 104 = unnilquadium, 107 = unnileptium, 114 = ununquadium, 126 = unbihexium with the equivalent symbols Unq, Uns, Uuq, Ubh.

This artificial system may look cumbersome and unnecessary for merely referencing the elements. The atomic number presently serves that purpose adequately. However, when discussions of chemical compounds involving these elements begin to appear in the literature, this system will become necessary for the names of compounds and their formulas.

One may always refer to an element by its number only. However, the optional system above provides an internationally accepted name.

Of course, once the claim of discovery of a new element and its proposed name is accepted by the scientific community, the Commission is ready to incorporate any name given by the discoverer.

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Autocrine growth factors and cancer

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The ability of cancer cells to produce and to respond to their own growth factors (autocrine secretion) has become a central concept linking oncogene and growth factor research. Oncogenes confer growth factor autonomy on cells not only by coding directly for autocrine peptide growth factors or their receptors, but also by amplifying the mitogenic signals generated by a growth factor at its receptor. Antagonists of positive autocrine growth factors can inhibit growth of cancer cells in experimental animals. Recently identified negative autocrine growth factors might themselves control aberrant cell growth.

AUTOCRINE secretion of growth factors is a concept which is emerging as a unifying theme in the search for the molecular and cellular basis of malignant transformation. This search has resulted in the merging of the previously separate fields of study of either oncogenes or peptide growth factors, as seen in the recent discoveries that oncogenes can confer growth factor autonomy on cancer cells¹⁻³. The relatively autonomous nature of malignant cells has been known for many years; that is, they require fewer exogenous growth factors for optimal growth and multiplication than do their normal counterparts⁴. To explain this phenomenon, it was suggested that cells could become malignant by the endogenous production of polypeptide growth factors acting on their producer cells via functional external receptors, allowing phenotypic expression of the peptide by the same cell that produces it⁵. This process has been termed 'autocrine secretion'⁶. The ability of oncogenes to make cancer cells autonomous of growth factors can now be explained by this autocrine hypothesis, particularly if the hypothesis is broadened to include the importance of receptors and post-receptor signal transduction as critical control elements in the normal mitogenic pathway. Here we summarize data supporting the original autocrine hypothesis and present a revised version to account for some significant new findings.

Evidence for the autocrine hypothesis

There is now much circumstantial and direct evidence to support the original hypothesis. Many types of tumour cells release polypeptide growth factors into their conditioned medium when grown in cell culture and these same tumour cells often possess functional receptors for the released peptide. The peptide growth factors that function via an autocrine mechanism in cancer cells include type α transforming growth factor (TGF- α), peptides related to platelet-derived growth factor (PDGF), bombesin and type β transforming growth factor (TGF- β). The action of each of these four peptides is mediated by a distinct membrane receptor, which in turn activates a post-receptor signalling mechanism and leads eventually to a mitogenic response. As discussed below, such a signalling pathway may be modified by oncogene expression at the receptor or post-receptor levels, as well as by alteration of the level of expression of the growth factor itself. Indeed, the ability of many oncogenes to confer growth factor autonomy on cancer cells seems to be related to their alteration of a receptor or of a post-receptor signalling pathway, rather than to a primary alteration in the synthesis and release of a specific growth factor.

The autocrine action of a growth factor in a cancer cell was first described in rodent cells transformed by either Moloney⁷ or Kirsten⁸ murine sarcoma viruses (Mo-MSV and Ki-MSV, respectively). The first peptides identified as participating in this transformation⁹ are structurally related to (but distinct from) epidermal growth factor (EGF) and are now called type α TGFs. The only known receptor for these peptides is the EGF receptor and all of their effects are thought to be mediated through this

locus. Many data indicate that human cancer cells produce and release TGF- α and have functional receptors for this peptide¹⁰⁻¹³. The relationship between release of TGF- α and transformation is clear, as a result of experiments with temperature-sensitive mutant rodent sarcoma viruses^{8,14,15}. In these experiments, TGF- α was not released into the medium unless cells were grown at a temperature permissive for transformation. Thus, the strict dependency of TGF- α secretion on the transformed state of these sarcoma cells suggests that viral oncogene products such as p37^{mos} and p21^{K-ras} control TGF- α synthesis at either the transcriptional or translational level. The recent cloning of both the human¹⁶ and rat¹⁷ genes for TGF- α will now enable investigation of the details of the actual molecular mechanisms involved. Transformation of rodent cells by either the Moloney, Harvey (Ha) or Kirsten viruses involves additionally the release of other peptide growth factors, in particular TGF- β (ref. 18) and PDGF-related peptides¹⁹. A direct mechanistic connection between transformation and the release of these other peptides has not yet been shown, although it is known that phenotypic transformation of non-neoplastic rat kidney (NRK) fibroblasts by growth factors requires the concerted action of at least three peptides, TGF- α , TGF- β and PDGF²⁰.

The experimental data providing the most evidence for the autocrine hypothesis come from the relationship of the oncogene product of simian sarcoma virus (SSV) and PDGF; the N-terminal 109 amino acid residues of the B-chain of PDGF are almost identical with the sequence of the transforming protein p28^{sis} of SSV^{21,22}. Production of PDGF-like molecules occurs in human osteosarcoma^{23,24} and glioma²⁵ cells, in several lines of simian virus 40 (SV40)-transformed cells^{19,26}, in mouse fibroblast 3T3 cells transformed by Mo-MSV and Ki-MSV¹⁹, as well as in the human T24 bladder cancer cell line expressing a mutated *ras* proto-oncogene¹⁹. The PDGF-like substances produced by all these tumour cells, except for the SSV-transformed cells, seem to be encoded by cellular genes. Many of the transformed cells producing PDGF-like peptides also have functional PDGF receptors^{19,27,28}; antisera to PDGF specifically block thymidine incorporation into DNA when added to cultures of 3T3 or NRK cells transformed by SSV²⁸. Furthermore, the ability of a series of SSV-transformed cells to grow in nude mice correlates with their ability to produce and release PDGF-related peptides; transformed cells secreting low amounts of PDGF grow poorly in nude mice, whereas cells secreting high amounts form large tumours²⁸.

Recent studies with PDGF-like peptides in smooth muscle cells from both young²⁹ and injured³⁰ animals now provide experimental evidence for an earlier suggestion⁶ that the autocrine mode of growth factor action is not limited to cancer cells. Although arterial smooth muscle cells isolated from adult rats do not produce significant amounts of PDGF-like peptides, the corresponding cells from either very young rats or rats with arterial wall trauma release biologically significant amounts of these peptides into their extracellular medium. Autocrine

mechanisms for self-stimulation may thus be of critical physiological importance during the repair of tissue injury, as in wound healing and during the explosive (and sometimes invasive) growth of the early embryo; only the inappropriate expression of an autocrine peptide may be pathological.

Most of the above data provide only circumstantial evidence for the biological significance of an autocrine mechanism of growth factor action in cancer cells. A critical experiment would be to control the growth of cancer cells by an extracellular antagonist of a presumed autocrine peptide. This has been accomplished recently³¹ with monoclonal antibodies to the tetradecapeptide bombesin, produced and released by most human small-cell lung cancers ('oat cell' cancers)³²⁻³⁴. High concentrations of bombesin occur in these highly malignant cells, which also have functional receptors for the peptide³⁵. Bombesin is a potent mitogen for 3T3 cells³⁶ and can stimulate clonal growth of small-cell lung cancer cells in serum-free medium³⁷. Monoclonal antibodies specific for the C-terminal region of bombesin not only inhibit the binding of bombesin to its receptor, but also inhibit markedly both the clonal growth of small-cell lung cancer cell lines *in vitro* and the growth of xenografts of these cells in nude mice³¹. These experiments provide a paradigm for other growth factors that may act via an autocrine mechanism.

Other autocrine mechanisms

As noted earlier, the autocrine action of an effector peptide may be amplified by mechanisms other than a concentration increase. For example, enhanced cellular responsiveness to a growth factor may result also from a change in the number or affinity of the receptors for the growth factor. Thus, very high numbers of EGF receptors occur in squamous carcinoma cells derived from human head and neck cancers³⁸, as well as in the intensively-studied A431 cell line^{5,39}, derived also from a squamous carcinoma. In A431 cells, a population of receptor molecules with extremely high affinity for EGF has been identified; this subset of receptors may be involved in the mitogenic response of cells to EGF^{40,41}. Such receptors are important, because monoclonal antibodies to them inhibit not only the proliferation of A431 cells in culture, but also their growth *in vivo* in athymic mice⁴². Another type of tumour cell in which a receptor abnormality seems to be important is the leukaemic adult human T cell. Unregulated expression of the receptor for interleukin-2 (IL-2, T-cell growth factor) may contribute to leukaemogenesis⁴³⁻⁴⁵, although at present there is little evidence for significant autocrine action of IL-2 itself in leukaemic T cells⁴⁶.

A further way to achieve an enhanced degree of signalling by the pathway normally activated by either TGF- α or EGF is to make the activation of the receptor independent of the effector; the *erb-B* oncogene may function in this manner to transform cells. The product of this gene has significant sequence homology to the transmembrane and cytoplasmic (tyrosine kinase) domains of the EGF receptor³, but the protein encoded by *erb-B* lacks the portion of the EGF receptor responsible for the extracellular binding of its ligand. In this case, the truncated receptor probably has the capacity to generate a mitogenic signal, independent of its ligand. Other oncogene products localized to the cell membrane (such as the *src* and *abl* proteins⁴⁷), as well as other growth factor receptors (such as the receptors for PDGF⁴⁸, insulin⁴⁹ and insulin-like growth factor I⁵⁰), also have intrinsic tyrosine kinase activity. Thus, the *erb-B*/EGF receptor homology may be a useful model for identification of other growth factor receptors as products coded by oncogenes.

In addition to the oncogenes related directly to either growth factors or their receptors, other sets of oncogenes confer growth factor autonomy via indirect mechanisms, such as the post-receptor signal transduction pathways that generate a mitogenic response in a cell after growth factor stimulation; that is, the events whereby changes in the membrane receptor are translated into activation of specific genes in the nucleus. Three oncogenes implicated directly in these signalling pathways are Ha-*ras*, *myc* and *fos*. The p21 product of the Ha-*ras* gene is localized on the

cytoplasmic side of the plasma membrane^{51,52} and binds GTP⁵³. Normal cellular p21 has a GTPase activity, greatly reduced when the transforming potential of p21 is activated by mutation^{54,55}. These data, together with the homology between the *ras* oncogene products and the G-protein regulators of adenylyl cyclase activity⁵⁶ (which require bound GTP for activity), implicate *ras* in transduction of the signal across the plasma membrane. Experiments showing GTP-binding activity of transforming Ha-*ras* p21 to be enhanced by EGF⁵⁷ (and presumably by TGF- α) further suggest that secretion of TGF- α by *ras*-transformed cells has a role in amplifying this signalling pathway.

In contrast to the *ras* p21 proteins, peptides encoded by the *fos*⁵⁸, *myc*^{59,60} and *myb*⁶¹ oncogenes are localized in the nucleus; furthermore, they are expressed differentially during cellular proliferation⁶²⁻⁶⁴ and differentiation⁶⁵⁻⁶⁷. Two lines of evidence link directly these oncogenes to signalling pathways for growth factors: first, treatment of cells with PDGF enhances transcription of both *myc*⁶² and *fos*^{63,68} genes; second, although mitogenic stimulation of normal fibroblasts by EGF requires PDGF⁶⁹, fibroblasts transfected with a cellular *myc* gene attached to a strong promoter no longer require PDGF to induce a 'competency' for replication (these cells undergo mitosis when treated with EGF alone)⁷⁰. Cells transfected with *myc*, but not their normal counterparts, can be stimulated to form colonies in soft agar in the presence of growth factors such as EGF⁷¹. Thus, constitutive expression of *myc* alters the sensitivity of a cell to growth factors. It is not known whether *fos* and *myb* confer similar cellular sensitivity to growth factors and if the peptide products of *fos*, *myc* and *myb* are induced in parallel, or form part of a sequential signalling pathway⁶³.

Negative autocrine growth factors

The signalling pathways activated by an autocrine peptide need not evoke a positive growth response, as seen most strikingly in the response of cells to TGF- β . This peptide was characterized initially by its ability to stimulate the growth of non-neoplastic fibroblasts as colonies in soft agar⁹. However, TGF- β can be also a potent inhibitor of the growth of many cells; in one case this negative action of TGF- β is clearly autocrine^{72,73}. TGF- β (or a closely related peptide) is released by confluent monkey kidney cells; these cells have receptors for TGF- β ⁷⁴ and respond with an inhibition of growth^{72,73}, suggesting that in certain cells endogenous TGF- β plays a role in cell-cycle regulation, specifically in the maintenance of a resting state. Picogram amounts of exogenous TGF- β can inhibit the growth of many types of cells⁷¹, including both neoplastic and non-neoplastic cells of either fibroblastic or epithelial morphology. The set of conditions in a cell determining whether the action of TGF- β is positive or negative for growth is complex and cannot be explained simply by differences in cell type, growth conditions or the concentrations of TGF- β . For example, rat fibroblasts transfected with the *myc* gene have a response to TGF- β dependent on the entire set of growth factors acting on the cell; in the presence of PDGF, picogram amounts of TGF- β stimulate anchorage-independent cell growth whereas the same amounts of TGF- β have a strong anti-proliferative effect on cell growth in the presence of EGF⁷¹.

A revised autocrine hypothesis

The growth inhibitory properties of TGF- β suggest that this peptide, purified to homogeneity⁷⁵⁻⁷⁷ and characterized in terms of its amino acid sequence (R. Derynck *et al.*, in preparation), represents a new class of autocrine negative growth factors. It has been suggested previously that a cell might produce its own specific growth inhibitory substances^{78,79}, but any strong experimental evidence based on studies with highly potent homogeneous peptides was lacking until it was found that TGF- β suppresses the growth of the same monkey kidney cells that produce it^{72,73}. Thus, the autocrine hypothesis may now be extended to include the concept that malignant transformation may be the result not only of excessive production, expression and action of positive autocrine growth factors, but also of the

failure of cells to synthesize, express or respond to specific negative growth factors they normally release to control their own growth. This loss of negative growth control may result from a biochemical lesion in either the growth inhibitor, its receptor or the post-receptor signalling pathway.

The development of antagonists of positive autocrine growth factors, suggested previously⁶, has provided a useful new approach to the control of growth of cancer cells. The newer

formulation of the autocrine hypothesis presented here now additionally emphasizes the importance of negative growth factors for this purpose. The new hypothesis is therapeutically optimistic as it renews interest in the possibility of restoring growth control to cancer cells by replacement of a deleted or defective growth inhibitor.

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ARTICLES

Narrow and variable lines in the ultraviolet spectrum of the Seyfert galaxy NGC4151

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The Seyfert galaxy NGC4151 observed when the nucleus is at a minimum has two emission lines of full width at half maximum <7 and 16 Å respectively and varying in intensity by a factor of three in 10 days. These lines are too narrow to be emitted by the whole broad-line region and must arise instead from two localized regions which have a special excitation mechanism, possibly a two-sided jet.

THE nature of the energy source in quasars and active galactic nuclei is still unknown. Information on the physical conditions near the energy source can be obtained from the analysis of the optical and ultraviolet spectrum of the dense gas in the nucleus. Our previous work on the time variations of the continuum

emitted by the central source^{1,2} and of the intensity and profiles of the emission lines³ has established that the line intensities vary in response to the variations of the central ionizing flux and that the broadest components of the lines vary with the shortest time scales⁴. As an example, the variable component

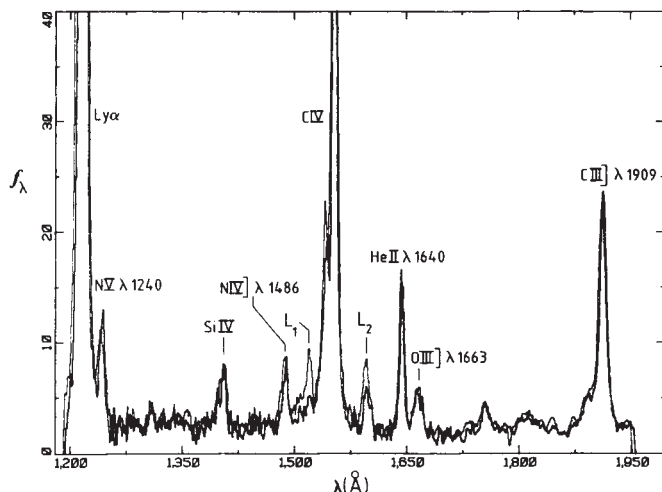


Fig. 1 The ultraviolet spectrum of NGC4151 between 1,200 and 1,950 Å in May 1981. Thick line, spectrum on May 12; thin line, spectrum on May 17. Each spectrum is obtained from two SW P images taken during the same IUE shift. Note that the spectrum has not changed in 5 days except at the positions of L_1 and L_2 . Ordinates in $10^{-14} \text{ erg cm}^{-2} \text{ s}^{-1} \text{ Å}^{-1}$.

of the C IV λ 1550 line has a full width at half maximum (FWHM) of $\sim 40 \text{ Å}$ and varies on a time scale of 10 days³. The details of the time variations can best be explained by the light travel time effects of the varying continuum as it spreads throughout a gaseous nebula where the velocity dispersion of the gas decreases from the centre outwards^{3,4}.

The discovery of two narrow emission lines on either side of C IV λ 1550 (FWHM corrected for instrumental profile < 7 and 16 Å respectively) and varying on a time scale of 5 days was therefore unexpected. These unidentified lines do not follow the behaviour of the lines emitted by the entire broad-line region, which suggests that they come from special locations in the broad-line region and have a special excitation mechanism. We describe here the analysis and interpretation of these two emission lines. Our investigation is restricted to the periods of minimum or 'low state' of the nucleus of NGC4151 when these lines can be best studied.

Low states and observations

The intensity of the ultraviolet continuum and of the C IV λ 1550 line in NGC4151 varies by a factor of ~ 3.5 (refs 2, 3). Sometimes, the nucleus goes into low states characterized by: (1) weakness of the wings of the C IV line and of the other weaker permitted lines and (2) low level of the ultraviolet continuum with $f_\lambda(1,440\text{--}1,470 \text{ Å}) \leq 4 \times 10^{-14} \text{ erg cm}^{-2} \text{ s}^{-1} \text{ Å}^{-1}$. The weakness of the C IV wings probably results from an insufficient photon flux.

Our earlier data set^{2,3} (taken between April 1978 and May 1980) showed NGC4151 in a low state only once, on 1980 April 21. Remarkably, low states have become more frequent between March 1981 and June 1984. The data analysed here were taken on 43 different days between 1981 March 26 and 1984 June 6. Each observation corresponds to 4 h of IUE²² time, sufficient to take two correctly-exposed spectra of NGC4151 in each of the two wavelength ranges of IUE (short wave (SW): 1,200–1,970 Å and long wave (LW) 1,920–3,190 Å). The characteristics of the 1981 spectra are given in ref. 5 and those of the other spectra remain to be described.

Special lines L_1 and L_2

Line intensities at low states. The special lines L_1 and L_2 are shown in Fig. 1. The two spectra, taken 5 days apart in May 1981, represent the fastest variation of L_1 and L_2 so far observed. Between April 1981 and June 1984, L_1 and L_2 have undergone several maxima and minima (Fig. 2a,b). The lines L_1 and L_2 were very weak (or undetectable) on 1981 March 26–30, 1981

July 16, 1984 March 30–April 2, whereas they were strong in 1981 May 17–June 8, 1984 March 6–18 and clearly detectable on 1984 June 2. Thus, L_1 and L_2 fade and then reappear several months later at the same wavelengths. At the same low-state epochs, the other lines remain constant, except for some small variations in the (weak) wings of C IV λ 1550 and the blue side of the narrow component of this line.

L_1 and L_2 are not caused by instrumental effects because: (1) The positions of the lines in the recorded spectrum or in the corresponding background subtracted to obtain the net spectrum are close neither to the resseau marks nor to the bright spots of the detector which all have well-known and catalogued positions⁶. (2) The spurious spectral anomalies which recur at fixed locations on the camera target and the fixed pattern noise are also well documented⁷. None of these spurious features occur at wavelengths close to those of L_1 and L_2 . (3) L_1 and L_2 do not appear in the spectra of standard stars^{8,9} routinely taken for calibration purposes and which have a signal-to-noise ratio similar to our spectra. (4) These lines have not been seen in various kinds of well-observed objects such as planetary nebulae, 3C273, BL Lac objects and others, the spectra of which have been scrutinized for new emission lines in the ultraviolet.

On the first ultraviolet medium resolution spectrum of NGC4151 ever taken (recorded during a rocket flight on 10 February 1978), a line, most probably L_2 , at 1,584–1,591 Å with an intensity of $73 \times 10^{-14} \text{ erg cm}^{-2} \text{ s}^{-1}$ (close to the maximum observed for L_2) was detected¹⁰. The continuum at 1,440–1,470 Å is $\sim 8.5 \times 10^{-14} \text{ erg cm}^{-2} \text{ s}^{-1} \text{ Å}^{-1}$, corresponding to a medium state and consistent with the fairly strong wings of C IV λ 1550 on that date.

The intensities of L_1 and L_2 at low states have been measured in the intervals 1,513–1,528 Å and 1,590–1,613 Å, respectively, by approximating the local continuum with a straight line. The intensities in 1981 and 1984 are plotted in Fig. 3a,b together with the continuum in the interval 1,440–1,470 Å. Variations by a factor of ≤ 4 have occurred, whereas during the same low states, the lines N IV λ 1486 and He II λ 1640 of similar width have varied by $< 20\%$, consistent with no variations (within our limits of experimental error).

There is some correlation between L_1 and L_2 variations, for example, the lines appear and disappear together. Their intensity (I) ratio $I(L_2)/I(L_1)$ is not constant but oscillates between 1.8 and ~ 4 . There may be some connection between the intensities of the lines and the continuum level, as the lines vanish below a certain value of the continuum, $f_\lambda(1,440\text{--}1,470) \sim 2.7 \times 10^{-14} \text{ erg cm}^{-2} \text{ s}^{-1} \text{ Å}^{-1}$.

The time delay, t_d , between the variations of L_1 and L_2 is the largest number of days by which the light curve of L_1 can be shifted (backward or forward) and still overlaps with the light curve of L_2 . Figure 3a,b gives $t_d < 10$ days, this limit originating mainly from the variations in May 1981.

Wavelengths and line widths. The wavelengths of L_1 and L_2 have been measured by fitting gaussian curves without removing the

Table 1 Observed and rest wavelengths of L_1 and L_2 (Å)

Epochs of observing	N IV λ 1486.5	L_1	L_2	He II λ 1640.5
1980 April 21	1,491.2 (1.3)	1,522.9 (1.4)	1,600.7 (1.5)	1,646.5 (1.5)
1981 May 17–June 8	1,491.0 (0.3)	1,523.5 (0.6)	1,599.7 (0.8)	1,647.0 (0.5)
1983 November 7–19	1,491.0 (0.4)	1,524.1 (0.8)	blend	1,647.0 (0.4)
1984 March 6, 10; June 2	1,490.5 (0.8)	1,523.5 (0.7)	1,598.3 (0.6)	1,646.4 (0.7)
Average	1,490.9	1,523.5	1,599.6	1,646.7
Rest wavelengths	1,486.1	1,518.5	1,594.4	1,641.4

The wavelength of L_1 and L_2 in four different years measured at those epochs or groups of epochs of low states when L_1 and L_2 are strong. Errors are shown in parenthesis. Before the measurements some of the spectra were shifted slightly in wavelength to have the peak of C III λ 1908.73 at 1914.95 on all spectra ($z = 0.00326$). The 6th line gives the average for the four different years with the same weight to all years; the last line gives the average rest wavelengths.

local continuum; the accuracy of the measurement decreases as the lines become weaker. There is, however, no sign that the measured wavelengths vary systematically with the line intensities. To explore the possibility of a secular trend of the wavelengths, we have selected for each year the low state days when the lines were stronger (Table 1). We find an upper limit of 0.10 on the relative change over 3 yr of the difference in wavelength between the C IV peak and either of the lines L_1 and L_2 .

The rest wavelengths obtained from the mean of the values in Table 1 and after correction for the systemic red shift are 1,486.1 Å and 1,641.4 Å for N IV]λ1486.5 and He II λ1640.5 respectively, and are 1,518.5 Å and 1,594.4 Å for L_1 and L_2 respectively, with an accuracy of ± 1.5 Å.

The FWHM of medium or strong L_1 and L_2 is ~ 11 and ~ 18 Å, respectively (without correction for the instrumental profile) and ≤ 7 Å and ≤ 16 Å respectively after this correction. The FWHM of the broad variable component of C IV λ1550 has been measured on the difference spectra obtained by subtracting a low state spectrum (specifically, from 1980 April 21) from the spectrum at different epochs of medium or high states. (For examples of such spectra, see refs 1, 3, 4.) We find that it depends slightly on the epoch and is most often between 45 Å (for example, 1978 December 9, 1980 May 15) and 40 Å (for example, 1978 October 19), thus appreciably larger than the FWHM of L_1 and L_2 . An important consequence of the narrowness of L_1 and L_2 is that these lines do not originate from the entire region that emits the broad variable component of C IV λ1550, but must come instead from a region or regions with a localized ionization and excitation mechanism.

High or medium states of the spectrum. When the nucleus is in a high or medium state, that is, 1978–1980 (except 21 April 1980), 1982, 1983 (except 15–19 November), L_1 and L_2 are not seen distinctly; they are blended with the wings of the C IV line. Their temporal behaviour at medium and high states is thus not well known except that: (1) L_1 and L_2 do not vary proportionally to the wings of the C IV line at their wavelengths and (2) there is no evidence so far that the lines (if they keep the same FWHM) ever become appreciably stronger than they can be at low states, for example, in 1981 May 17–June 8. This second point was verified by subtracting a fraction (between 1 and 2.5) of the lines L_1 and L_2 as they are on 23 May 1981, from spectra at different high states. The subtraction produces a minimum at the position of L_1 and L_2 , indicating that these lines do not increase substantially above twice their value of 23 May 1981 regardless of the total intensity of C IV or of the continuum. Thus, either the lines L_1 and L_2 do not respond to the variations of the ionizing continuum responsible for the variations of the bulk of C IV λ1550, or they stay constant or decline when the continuum is intense because the element which they represent enters a higher state of ionization.

In summary, when the nucleus passes from low states to high states, L_1 and L_2 do not follow the general increase of intensity of the permitted lines accompanying the increase of the continuum flux. At low states, they can vary by a factor up to 4 whereas the other lines stay constant. (If the full width at half intensity of L_1 and L_2 increases at medium and high states, L_1 and L_2 can blend with the wings of C IV λ1550 and could increase significantly in intensity without being detectable as separate lines.)

No attempt has been made to measure the intensities of L_1 and L_2 at high and medium states. Measurements of L_1 (Fig. 3c) for February 1982 and 4–11 November 1983 which are epochs when the nucleus was almost in low states are considered tentative because the wing of C IV λ1550 makes the definition of the 'local continuum' rather uncertain.

Feature L'_2 . L'_2 is most often seen as a narrow isolated line, but sometimes appears blended with a feature L'_2 varying in intensity and possibly also in width and in wavelength (Fig. 2c). In 1981, L'_2 was almost absent and was rather weak in 1984; it may have increased and faded again in the meantime, suggested by the evolution of the spectrum from 1982–1983 (Fig. 4) when the

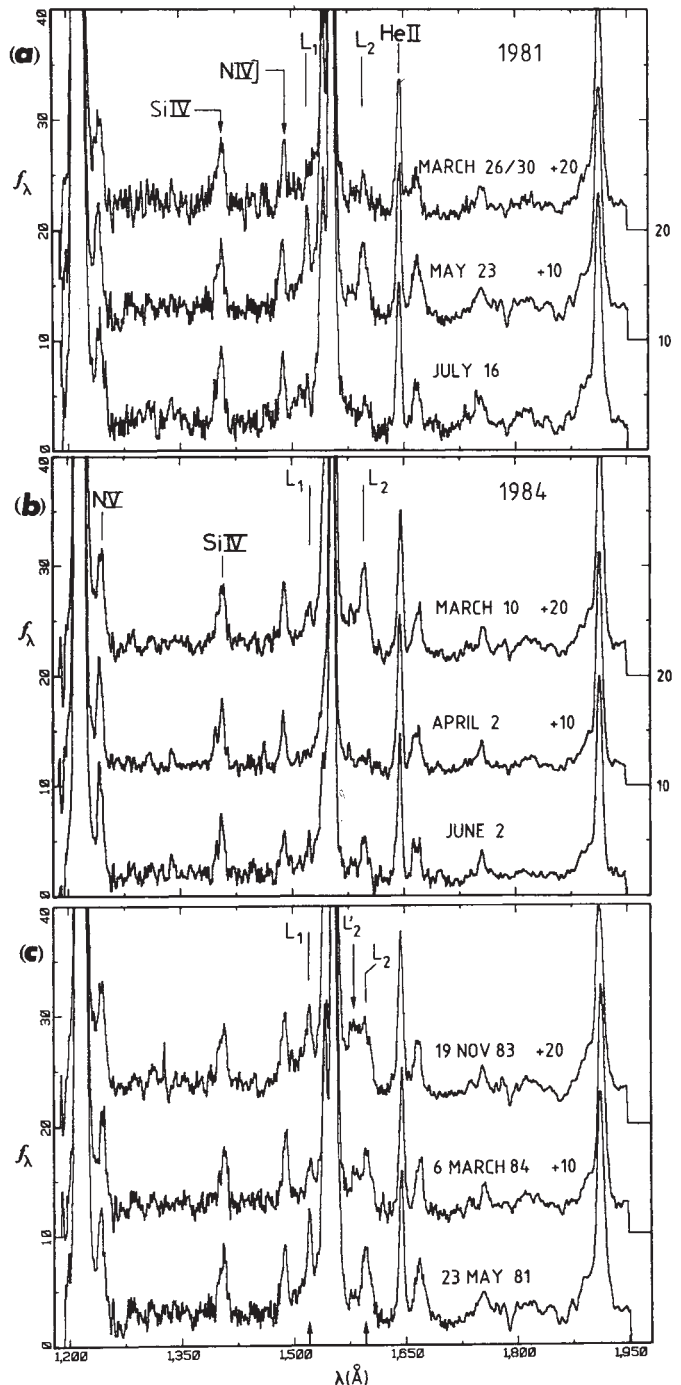


Fig. 2 Examples of NGC4151 spectra at 8 epochs, all low states. *a, b*, Spectra arranged in chronological order showing the variations of the lines L_1 and L_2 . *a*, 1981 March 26/30 shifted by +20, May 23 shifted by +10 and July 16. *b*, 1984 March 10 shifted by +20, April 2 shifted by +10 and June 2. Ordinates in 10^{-14} erg cm $^{-2}$ s $^{-1}$ Å $^{-1}$. *c*, Three low-state spectra arranged by increasing contamination of L_2 by the feature L'_2 . When L'_2 is nearly as large as, or larger than L_2 (for example, 1983 November 19) no attempt has been made to measure $I(L_2)$. The spectrum of 19 November 1983 has been shifted by +20 and that of 6 March 1984 by +10. Ordinates in 10^{-14} erg cm $^{-2}$ s $^{-1}$ Å $^{-1}$.

nucleus was at medium or high states. The feature L'_2 seems to have increased from February 1982, reached a maximum on 6 November 1982 then decayed in 1983, suggesting that a substantial fraction of the red wing of the C IV line was contributed to by a single red-shifted cloud. When L'_2 is almost as strong as L_2 (or stronger) we have not measured the intensity of L_2 because it is not possible to separate it from L'_2 . When L'_2 is weaker, as

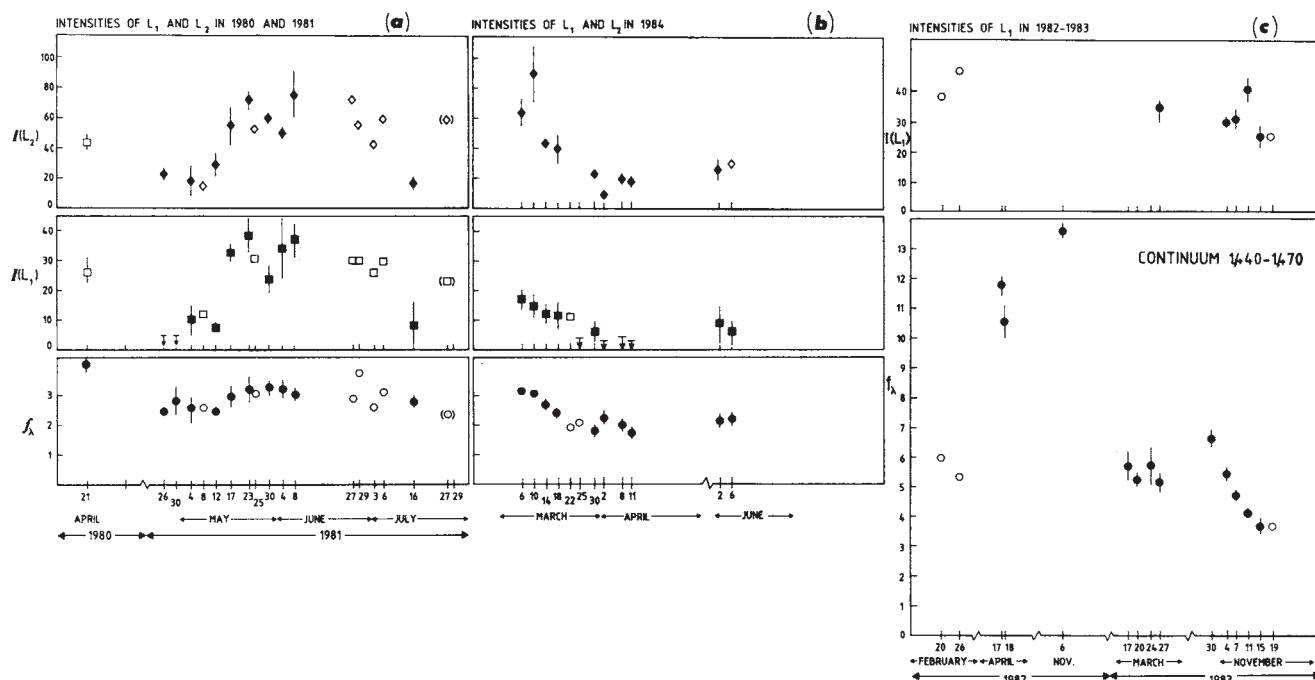


Fig. 3 The continuum in the window 1,440–1,470 Å and line intensities at the 43 epochs at which NGC4151 was observed between 26 March 1981 and 6 June 1984. *a, b*, Observations in 1981 and 1984. The nucleus was in a low state at all epochs of observations. The low state 1980 April 21 is added to *a*. *c*, The continuum in 1982 and 1983. Measurements of L_1 in February 1982 and 4–11 November 1983 are tentative. Closed circles, average of the values obtained from two different images taken during same shift; extremities of the error bars represent the individual measurements; open circles, value measured on one spectrum only; ordinates, f_A is in 10^{-14} erg cm $^{-2}$ s $^{-1}$ Å $^{-1}$; $I(L_1)$ and $I(L_2)$ are in 10^{-14} erg cm $^{-2}$ s $^{-1}$.

on 1984 March 6, the local continuum used to measure L_2 is the one underlying both L_1' and L_2 .

Identifications

Several mechanisms of fluorescence give lines in the ultraviolet such as the excitation of H_2 by $Ly\alpha$ (ref. 11) and the excitation of CO and Fe II lines by the C IV $\lambda 1550$ doublet^{12,13}, but none of the lines coincide with L_1 and L_2 . We could not find any convincing evidence for L_1 and L_2 in line lists of objects such as planetary nebulae, the Sun¹⁴ or RR Tel¹⁵. Nor could we identify any new fluorescence mechanisms using the Revised Multiplet Table and other line lists^{16,17}.

We do not agree with the identification with O III proposed for L_2 (ref. 11) as the excitation of so high a term is difficult. Even if L_1 and L_2 are emitted by ions at the red shift of NGC4151, the problem of explaining the remarkable temporal behaviour and narrow width of the lines will remain.

It is important to search for L_1 and L_2 in other galaxies, as the existence of lines with the same behaviour and at the same wavelengths would indicate that these lines are transitions at rest with respect to the nucleus and not shifted components of C IV $\lambda 1550$. Weak features have been noted occasionally in the wings of the C IV line but none are as distinct or as narrow as L_1 and L_2 in NGC4151. A broad weak feature near $\lambda_{rest} = 1,588$ Å in MCG-2-58-22, NGC5548 and NGC7469 has been found and identified tentatively with multiplets of Si I (ref. 18). From our 26 spectra of NGC5548 (ref. 19), there is some weak evidence for a broad, possibly double, low signal-to-noise feature in the range $\lambda_{rest} \sim 1,580$ –1,610 Å. The spectrum of F9 during its minimum of October 1984 shows a weak narrow feature at $\lambda_{rest} = 1,588 \pm 1.5$ Å. Another weak feature appears in the spectrum of NGC5548 as a plateau or a change of slope near $\lambda_{rest} = 1,516 \pm 4$ Å and could be caused by Si II absorption at 1,521 Å.

The positional coincidence of the two features near 1,516 Å and 1,580–1,610 Å in NGC5548 with L_1 and L_2 in NGC4151 is not by itself sufficient to establish their identifications with L_1

and L_2 , because other processes (for example, individual large clouds in the broad-line region; multiplets of Fe II or other unidentified transitions) can produce features at these wavelengths which would approximately follow the behaviour of the other broad emission lines.

L_1 and L_2 as components of C IV $\lambda 1550$?

The lines L_1 and L_2 in NGC4151 are too narrow to be emitted by the whole broad-line region but must originate instead from two localized regions where the gas is ionized and excited by a mechanism other than photoionization by a roughly isotropic central continuum source.

L_1 and L_2 may be components of C IV $\lambda 1550$ emitted by two regions where the gas has a line-of-sight velocity of $-6,100$ and $+8,500$ km s $^{-1}$ respectively with respect to the systemic velocity. No components at these velocities are observed in $Ly\alpha$, C III $\lambda 1909$ and Mg II $\lambda 2798$. But in collisionally ionized plasmas (for example, stellar chromospheres and transition regions) C IV $\lambda 1550$ may be by far the strongest line and these non-detections may be explained by a combination of a high temperature, $T > 30,000$ K and a high electron density, $n_e > 10^9$ cm $^{-3}$, in the regions emitting L_1 and L_2 .

The maximum values of $I(L_1)$ and $I(L_2)$ are 35×10^{-14} and 70×10^{-14} erg cm $^{-2}$ s $^{-1}$, respectively. The mass of gas emitting L_2 at its maximum, calculated assuming normal abundances, a distance of 10 Mpc, C all in C $^{+3}$ and $T_e \sim 3 \times 10^4$ K is $1.5 \times 10^5 n_e^{-1}$ in $M_\odot$ or $\sim 10^{-4} M_\odot$ for $n_e \sim 10^9$ cm $^{-3}$.

There are several kinematic interpretations if L_1 and L_2 are components of C IV $\lambda 1550$, two of which are discussed briefly below:

Free-flying clouds. The regions emitting L_1 and L_2 could be emitted by two isolated clouds which are ejected, orbiting around the central object, or free falling. In ejected clouds, the distance (perpendicular to the plane of the sky) travelled in 3 yr by clouds leaving the nucleus with a line of sight velocity of $\sim 7,000$ km s $^{-1}$ is 7×10^{16} cm, several times the radius of the broad-line region. If these clouds are photoionized by the central source, an unob-

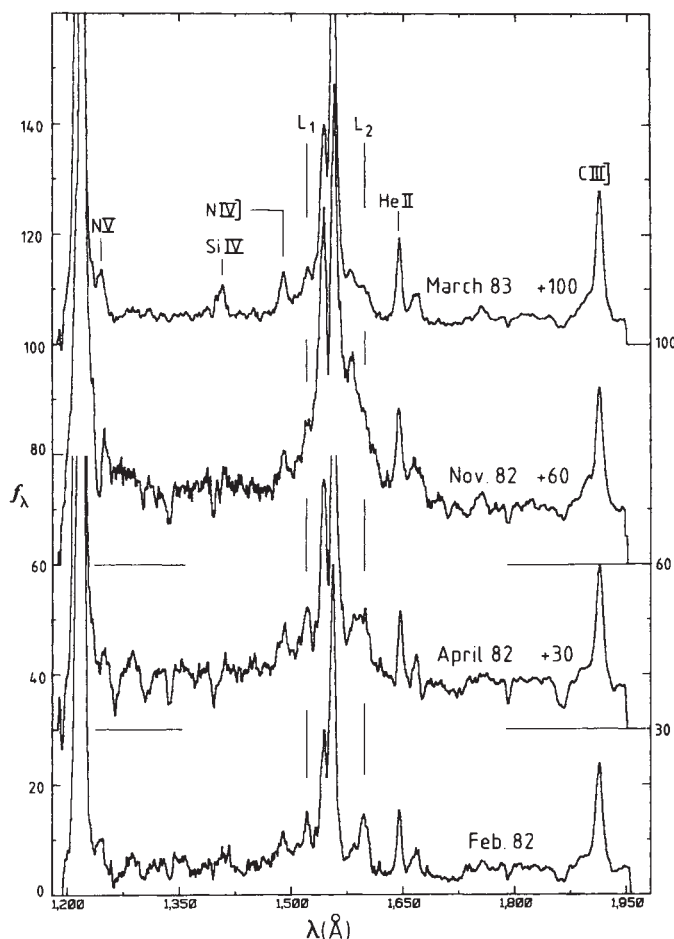


Fig. 4 The evolution of the spectrum of NGC4151 between February 1982 and March 1983. During that period the nucleus was found in medium and high states only. The spectra shown are the average of all the individual spectra taken the same month (the days of observations are found in Fig. 3c). The spectra of March 83, November 82 and April 82 have been shifted by +100, +60 and +30, respectively. Ordinates in 10^{-14} erg $\text{cm}^{-2} \text{s}^{-1} \text{\AA}^{-1}$.

served gradual and monotonic change of the intensity of L_1 and L_2 would be expected. Alternatively, the clouds could be in an approximately circular orbit. A minimum value of the trajectory radius, R , can be calculated from the fact that the relative variation of the line of sight velocity (v) is $dv_{\text{obs}}/v_{\text{obs}} < 0.10$ in 3 yr. Assuming that the clouds are now travelling at $\sim 45^\circ$ from the direction of the line of sight, $R \geq 8 \times 10^{17}$ cm, that is, 25 times further out than the normal clouds in the broad-line region. A third possibility is that the two clouds are falling towards a central object of mass $\sim 10^9 M_\odot$. The limit on the change in velocity in three years quoted above gives a limit to the acceleration undergone by the clouds and thus a lower limit to their radial distance. We find $R > 4 \times 10^{17}$ cm. All these possibilities

are inconsistent with the delay of < 10 days between the variations of L_1 and L_2 as this implies a line of sight separation of < 10 light days or $\sim 3 \times 10^{16}$ cm.

Bi-sided jet. Another possibility is that the special regions emitting L_1 and L_2 are associated with a two-sided jet similar to SS433. In this case, L_1 and L_2 would be emitted by gas in a constant velocity flow. Let us call θ the angle between the direction of the jet and the line of sight, d_1 and d_2 the distances of the regions emitting L_1 and L_2 from the origin of the jet and assume that the energy is carried along the jet at a velocity v . The time delay between the observed responses of L_1 and L_2 is $t_d = (d_2 - d_1)v^{-1} + (d_2 + d_1)c^{-1} \cos \theta$. The minimum delay (d) is for $d_1 = d_2$ and the observed upper limit of 10 days gives an upper limit of $(5/\cos \theta)$ light days on the mean distance of the regions emitting L_1 and L_2 to the centre. For comparison, the bulk of the broad variable component of C IV $\lambda 1550$ comes from a region spread between ~ 10 and 20 light days from the centre³. Thus, for $\theta < 60^\circ$ the regions emitting L_1 and L_2 are closer to the centre than is the broad-line region.

If the asymmetry of L_1 and L_2 in velocity shift is now interpreted as resulting from the transverse Doppler effect and the gravitational red shift can be ignored, and if the regions emitting L_1 and L_2 are moving at the same speed in opposite directions, then one obtains $\beta = v/c = 9.5 \times 10^{-2}$ or $v = 28,500 \text{ km s}^{-1}$ and $\theta = 75^\circ$. The jet would then lie almost in the plane of the sky, which would imply a large misalignment between the angular momenta of the massive central object and of the galaxy, as indicated by the radio jet.

The intensities of L_1 and L_2 are expected to vary in response to the variations of the amount of energy carried by the jet. Although a symmetrical jet produces identical variations of L_1 and L_2 , changing inhomogeneities in the gas in the regions emitting L_1 and L_2 can superpose different variations of L_1 and L_2 , the result being a similarity of the main features but not of the details of the variations of L_1 and L_2 , which is consistent with our observations.

Conclusions

The two narrow and variable emission lines on either side of the C IV $\lambda 1550$ line do not follow the behaviour of the broad emission lines emitted by the ensemble of dense clouds forming the broad-line region. They must therefore originate from small regions excited by a mechanism other than photoionization by a more or less isotropic central continuum source. An attractive (but speculative) possibility is that the regions emitting these lines (which, at present, remain unidentified) are associated with a two-sided jet; either a jet of relativistic particles which could be the inner section of the radio jet observed on a scale of $0.1 \times 5 \text{ arc s}^{20}$, or the non-relativistic jets which in the models of active galactic nuclei based on strong thermal winds can be collimated in two opposite directions²¹.

Further observations of NGC4151 should be aimed at measuring or obtaining stringent upper limits to the secular change in wavelengths of L_1 and L_2 and to the time delay between the variations of the line intensities. At the same time, the search for lines similar to L_1 and L_2 in other galaxies should be pursued actively.

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Limited diversity of the rearranged T-cell γ gene

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The immunoglobulin-related, T-cell specific γ gene is rearranged in a wide variety of murine T lymphocytes. We detected γ -gene transcripts in all cloned cytotoxic T lymphocytes examined but in only 1 of 11 T-helper cell lines or hybridomas. Although in cytotoxic T cells, the rearranged γ gene seems to have been assembled from the same germ-line variable and joining gene segments, the transcribed gene exhibited distinct sequence diversity near the junction between these segments.

THE populations of B and T lymphocytes that collectively constitute the immune system share many properties. Both populations are made up of many different clones, each distinguished by its ability to react specifically with only one or a few out of a practically limitless number of foreign antigens. The specificity of B cells for particular antigens results from clonally distinctive amino-acid sequences in the variable (V) domains of the heavy and light chains of the surface immunoglobulin molecules that serve as antigen-specific receptors for these cells. Thus, each clone of B cells normally produces two distinctive V domain sequences, one for its immunoglobulin heavy chains and the other for its light chains. T cells, like B cells, have surface antigen-specific receptor molecules also made up of two polypeptide chains¹⁻³. These T-cell receptor chains, α and β , are also clonally diversified by virtue of variable amino acid sequences in the N-terminal domain of each chain⁴⁻¹³. However, analysing a clone of cytotoxic T lymphocytes (CTLs) in which the genes for the α - and β -subunits are expressed, we found that there is also expressed a similar third gene, represented by cDNA clone pHDS4/203 (refs 10 and 11), hereafter called the γ gene.

This gene has many properties in common with α and β genes which include: (1) assembly from gene segments resembling the gene segments for immunoglobulin V, joining (J) and constant (C) regions; (2) rearrangement and expression of these gene segments in T cells and not in B cells; (3) low but distinct sequence homology to immunoglobulin V, J and C regions; (4) other sequences reminiscent of the transmembrane and intracytoplasmic regions of integral membrane proteins; (5) a cysteine residue at the position expected for a disulphide bond

linking two subunits of a dimeric membrane protein molecule. The function of the γ gene is unknown, but because of its shared properties with the α and β genes and the intriguing finding of three, rather than two, expressed V-region gene segments in a single clone of T cells, we seek here to learn more about its properties. We show that the gene is assembled in diverse T cells from predominantly the same V and J gene segments but that it is nonetheless diversified due to sequence variations in the region of the junction of its rearranged V and J gene segments; although the rearranged γ gene is transcribed in all the cloned CTLs examined, it seems to be transcribed only in infrequent T-helper cell lines and hybridomas. This suggests that the γ -gene product participates in determining the specificity of CTLs.

Rearrangements in T-cell lines

The germ-line configuration of the γ gene is rearranged in CTL clone 2C but not in myeloma cell lines¹⁰. To determine if rearrangement occurs in other types of T cells, we examined DNA from various T-cell lines using separate probes for the V and C regions of the γ gene. We also used a full-length (V + C) β -gene probe (pHDS11) to compare the variability of the rearrangements of the β gene with that of the γ gene. Consistent with previous findings¹⁴⁻¹⁶, a unique rearrangement pattern for the β gene was observed in almost all of the T-cell clones examined (Fig. 1a). The exceptions were CTL clones 2.1.1 and 1.5.2 which exhibited indistinguishable patterns; these clones are of BALB.B origin and are specific for the same antigen (L^d), but each arose in a different mouse.

Fig. 1 Southern blot analyses²⁶ of DNA from various T-cell lines. DNA was isolated from four alloreactive BALB.B CTL clones²⁷ (2C, 2.1.1 and 1.5.2, anti-L^d; G4, anti-D^d), three T-helper (T_H) hybridomas derived from a BW5147 × B10.A spleen cell fusion²⁸ (14; anti-GAT; 461 anti-dinitrophenyl ovalbumin; 2B4 anti-pig cytochrome c) and three BALB/c T-cell lymphomas²⁹ (88, 89 and S49). Blots were hybridized with the following ³²P-labelled, nick-translated probes (ref. 10): **a**, total insert of cDNA clone pHDS11 (cDNA clones were derived from the cDNA library of CTL clone 2C), corresponding to V + C regions of the β -chain gene; **b**, an *Ava*I fragment containing 840 bp at the 3' end of the pHDS4 insert, corresponding to the C region of the γ gene; **c**, an *Ava*I fragment containing 450 bp at the 5' end of the pHDS203 insert, corresponding to the V region of the γ gene.

Methods. DNA was digested with *Pvu*II (**a**) or *Eco*RI (**b**, **c**), electrophoresed through 0.8% agarose gels and blotted onto nitrocellulose. Blots were hybridized at 42 °C in 50% formamide and 5 × SSC and filters were washed at 65 °C in 0.2 × SSC.

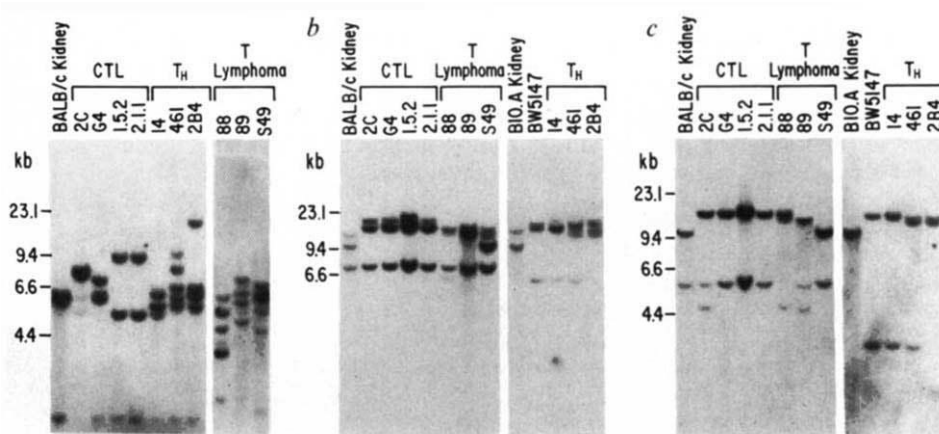
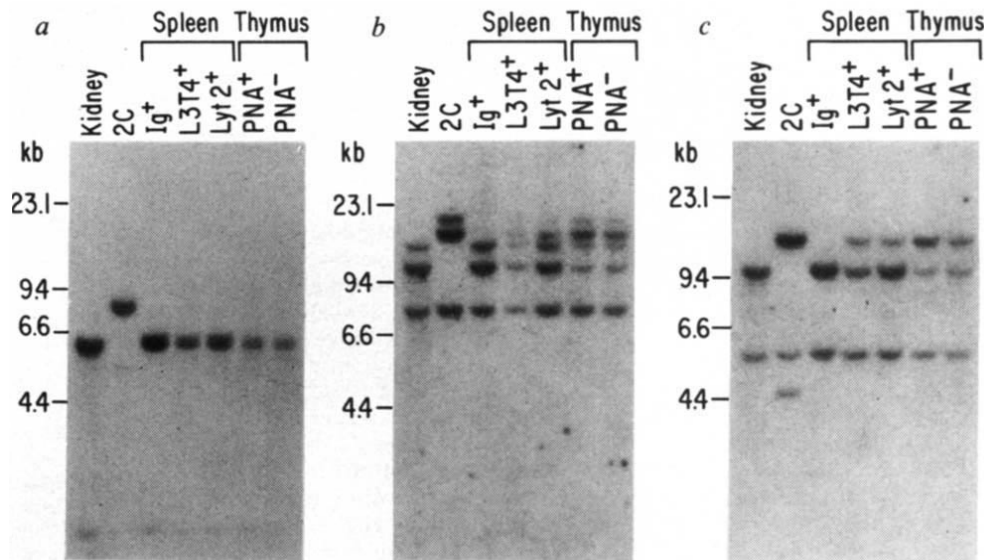


Fig. 2 Southern blot analyses of DNA from spleen and thymus subpopulations. DNA from subpopulations of BALB/c splenic lymphocytes and thymocytes were examined by hybridization with the following probes: a, $V_{\beta} + C_{\beta}$; b, C_{γ} ; c, V_{γ} .

Methods: Surface immunoglobulin positive cells (sIg⁺) were obtained by panning of BALB/c splenic lymphocytes (refs 30, 31) on plates pre-coated with rabbit anti-mouse immunoglobulin. Non-adherent cells were incubated with rat anti-Lyt-2 monoclonal antibodies (a mixture of monoclonal antibodies 2.43.5, 3.155.2, 3.239.2, 3.168.8, all provided by Dr Frank Fitch; ref. 32) or a rat anti-L3T4 monoclonal antibody (GK1.5, provided by Dr Katherine Wall, ref. 33) for 30 min. After washing, cells were incubated on plates pre-coated with rabbit anti-rat immunoglobulin and the adherent cells (Lyt 2⁺ or L3T4⁺) were recovered. BALB/c thymocytes were separated into mature and immature subpopulations by agglutination with peanut agglutinin (PNA⁻ mature; PNA⁺ immature)³⁴. DNA was extracted from the enriched subpopulations, digested with *Pvu*II (a) or *Eco*RI (b, c), and electrophoresed at approximately equivalent concentrations through 0.8% agarose gels. DNA was blotted onto nitrocellulose and hybridized as described in Fig. 1 legend.



In contrast to the highly diverse rearrangement patterns obtained with the β -gene probe, all CTL clones exhibited the same rearrangement pattern with the γ -gene probe, well illustrated by clone 2C. When *Eco*RI-digested clone 2C DNA was hybridized with the probe for the γ -gene C region, two rearranged bands of 16 and 22 kilobases (kb) and one 7.5-kb germ-line band were observed (Fig. 1b). We conclude that the 16-kb band contained the rearranged and expressed γ gene because it was the only C_{γ} -positive band in clone 2C that also hybridized with the probe for the V region of the γ gene (Fig. 1c). The rearranged 16-kb fragment arose from the joining of a J-C segment in the 10.5-kb germ-line fragment with a V segment contained in the 10.8-kb germ-line fragment¹⁷. As neither the C_{γ} -positive 10.5 nor the V_{γ} -positive 10.8-kb germ-line *Eco*RI fragments remain in clone 2C DNA, the V and C (or J-C) copies on the homologous chromosome must also have undergone rearrangement in this CTL line. The 10.5- and 10.8-kb gene segments are also joined non-productively in CTL clone 2C (see cDNA clone pHDS34, below).

The nature of the rearrangement(s) responsible for the disappearance of the C_{γ} -positive 13.4-kb germ-line band and the appearance of the 22-kb C_{γ} -positive V_{γ} -negative band is unknown, but both copies of the 13.4-kb fragment must also have undergone rearrangement. The V_{γ} gene probe also detected an additional band (5 kb) in clone 2C DNA absent in kidney DNA and possibly representing yet another rearrangement (Fig. 1c).

As shown in Fig. 1b, c, the rearrangement pattern obtained with the γ -gene probes in the other CTL clones was the same as in clone 2C, suggesting strongly that the same germ-line V and C (or J-C) γ -gene segments are rearranged in CTLs having different specificities. The rearrangement patterns of the T-helper hybridomas varied; T-helper 14 was similar to the parental line BW5147 although T helpers 461 and 2B4 each had a rearranged fragment distinct from that of BW5147. Each of the three BALB/c-derived T lymphomas (88, 89 and S49) also displayed a slightly different rearrangement pattern. The diversity observed with the γ -gene probe was much less than with the β -gene probe (Fig. 1a), consistent with the suggestion that only a very limited number of germ-line gene segments are used for γ -gene rearrangements.

Rearrangements in T-cell subpopulations

To determine whether the rearrangements observed are peculiar to cultured T-cells, we analysed DNA from several spleen and

thymus subpopulations. Southern blot analysis with the β -gene probe revealed a decrease in the intensity of the unrearranged 6.0-kb fragment in *Pvu*II-digested DNA from the splenic T-cell subpopulations and thymus subpopulations when compared with similarly digested DNA from kidney or B-cell preparations (Fig. 2a). As expected from the marked clonal variability in β -gene rearrangements (Fig. 1a), however, well-defined rearranged β -gene bands were not detected in these highly polyclonal T-cell populations.

In contrast, hybridization with the C_{γ} and V_{γ} probes displayed distinct and consistently rearranged fragments in all T-cell populations (Fig. 2b, c). In fact, the rearranged fragments in these populations and in all of the CTL lines examined seemed indistinguishable, containing both the rearranged fragment (16 kb) with $V_{\gamma} + C_{\gamma}$ sequences and the C_{γ} -positive/ V_{γ} -negative rearranged 22-kb fragment. Thus, even in the highly polyclonal T-cell populations, the γ -gene rearrangements are highly restricted and primarily involve the joining of a single V_{γ} to a single C_{γ} gene segment.

Like the β -chain gene, the γ -gene rearrangement must occur early in T-cell development as it was seen in both mature (PNA⁻) and immature (PNA⁺) thymocytes. In addition, CTLs and T-helper cells seem to exhibit γ -gene rearrangement as both the Lyt-2⁺ and L3T4⁺ subpopulations contained rearranged bands. It is not clear whether the persistent germ-line bands (C_{γ} region at 10.5 kb and V_{γ} region at 10.8 kb) in the heterogeneous T-cell preparations are due to unrearranged γ -gene segments in some T cells, or to the presence of some contaminating B cells, or other non-T cells that do not rearrange this gene.

Transcription

The γ gene is transcribed and gives rise to a 1.5-kb poly(A)⁺ RNA component in CTL clone 2C (ref. 10). Although this gene is rearranged in the other three cytolytic T-cell clones and presumably in some helper T cells (as T-helper hybridomas and the L3T4⁺ spleen cell subpopulation seem to exhibit rearrangements), it remained to be determined whether the γ gene is transcribed in all of these cells. Northern blot analyses¹⁸ of poly(A)⁺ RNA from two B-cell lymphomas, four CTL clones and two T-helper hybridomas were therefore performed with the C_{γ} probe (Fig. 3b) and, for comparison, with the β -gene probe (Fig. 3a). The cloned CTL and T-helper hybridomas contained approximately equal levels of transcript (1.1 and 1.3 kb) hybridizing to the β probe. In contrast, γ -hybridizing transcript was detected in four out of four CTLs, but in only one

of the two T-helper hybridomas and in none of five additional T-helper hybridomas and four L3T4⁺, Lyt-2⁻ T-cell lines (data not shown). Thus, transcription of the γ gene was seen consistently in CTLs but only rarely in T-helper cells or hybridomas. As reported previously¹⁰, no transcripts of either the β or γ gene were observed in the two B-cell lymphomas.

V-J junctional variability

To confirm that different CTL clones express the same germ-line V and C gene segments and also to search for clonal diversity, we isolated and sequenced three different cDNA clones from two different CTL clones. Two of the cDNA clones (pHDS4/203 and pHDS34) were isolated from CTL clone 2C (alloreactive, BALB.B anti-L^d)¹⁰ and the third (DFL12) from a cDNA library of CTL clone DFL12, which arose in a mouse of the DBA/2 strain and is specific for the fluorescein group in the context of the D^d class I molecule¹⁹.

We have shown previously that cDNA clone pHDS4/203 is composed of three regions (V, J and C) homologous with the corresponding regions of genes for immunoglobulins and α - and β -chains^{10,11}. As shown in Fig. 4, the nucleotide sequences of the three γ -gene cDNA clones are strikingly similar; indeed, the 5'-untranslated and V-region sequences of these clones are identical. The base-pair (bp) differences between 2C and DFL12 in the C and 3'-untranslated regions could be due to strain polymorphism (BALB.B versus DBA/2), the use of different C-region genes or somatic mutation. The latter possibility seems unlikely because no base-pair differences were found in the V region, where somatic mutation should be more common²⁰. Clone pHDS34 also contains a deletion of 30 bp (883-912), encoding the short peptide sequence that links the C domain with the transmembrane peptide. As this sequence is encoded by its own exon (ref. 17) and the extent of the deletion corresponds exactly to the ends of this exon, the poly(A)⁺ RNA corresponding to the pHDS34 cDNA clone probably arose either from a deletion of the exon at the DNA level or by skipping the exonic sequence during RNA splicing.

Apart from this deletion, the most striking differences between the γ -gene clones pHDS4/203, pHDS34 and DFL12 are at the boundary between the V- and J-encoded regions. Here, DFL12 has a deletion of the 9 bp coding for the tripeptide Trp-Met-Arg in pHDS 4/203 and pHDS34 has three consecutive cytidines (corresponding to a codon for Pro) in place of a pentanucleotide ATGAG, which corresponds to the codon for Met and the first two bases of an Arg codon in pHDS4/203. Thus, the polypeptide chain encoded by DFL12 differs substantially from that encoded by pHDS4/203 in the region of the V-J junction, whereas pHDS34 is in a wrong reading frame after the V-J junction and can specify only a V-encoded protein fragment. The junctional differences observed between pHDS4/203 and DFL12 could be attributed to polymorphism between the BALB.B-derived CTL clone 2C and the DBA/2-derived CTL clone DFL12; however, the finding that two cDNA clones (pHDS4/203 and pHDS34) from the same cell line (clone 2C) also differ at the V-J junction suggests strongly that such variability is a consequence of either somatic joining events, or the use of different diversity (D) or J gene segments, or terminal transferase activity²¹.

Discussion

The existence on T cells of antigen-specific receptors with immunoglobulin-like α - and β -subunits leads to the expectation that a T cell, like a B cell, should express only two V domains, one for each receptor subunit. However, an extensively studied clone of CTLs, 2C, expresses an additional gene, γ , (identified previously as cDNA clone pHDS4/203; refs 10, 11) that is also immunoglobulin-like; for example, it has ~20% sequence homology with immunoglobulin V and C domains and its 5' (amino-terminal) region codes for all of the invariant residues found in every heavy-chain V, light-chain V, V α and V β domain sequenced to date (J. Novotny, in preparation). To learn about possible functions of the γ gene, we have here evaluated the

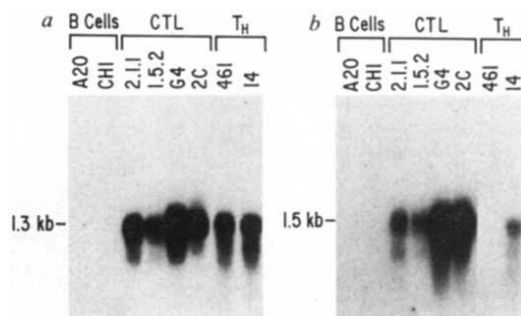


Fig. 3 RNA blot analyses (ref. 18) of poly(A)⁺ RNA from various B- and T-cell lines. RNA from B-cell lymphomas A20-2J and CH1, CTLs 2C, G4, 1.5.2 and 2.1.1 and T-helper hybridomas 14 and 461 were hybridized with the following probes: a, V β + C β ; b, C γ . **Methods.** Approximately 1.5 μ g poly(A)⁺ RNA was denatured with glyoxal and electrophoresed through 1% agarose gel in 10 mM sodium phosphate buffer, pH 6.5. RNA was blotted onto nitrocellulose and hybridized as described in Fig. 1 legend with ³²P-labelled nick-translated probe for V β + C β (a). The same filter was washed in boiling water at 100 °C for 5 min and hybridized with the probe for C γ (b).

extent of its variability using V and C-region hybridization probes and analysing DNA from diverse sources. We find γ -gene rearrangements in a wide variety of T cells (CTLs, T-helper hybridomas and polyclonal T-cell populations from spleen and thymus). The rearrangement patterns were identical in all CTLs, as though primarily the same V γ gene segment was joined to the same J γ gene segment.

In agreement with this suggestion, three cDNA clones (from two CTLs of different specificities and different mouse strains) had identical 5' V-region sequences and identical J-region sequences (Fig. 4), but these clones differed in sequence from each other near the V- and J-region junction. These clonal differences probably reflect junctional variability due to V-J joining events, the use of different D-gene segments, or terminal transferase activity.

These γ -gene properties demonstrate its marked resemblance to the genes encoding immunoglobulin λ chains in inbred mice²². The V region of each type of λ chain is encoded essentially by a single V and a single J gene segment and these chains similarly exhibit diversity at the V-J junction. Moreover, amino acid replacements at this junction can have a pronounced effect on ligand binding activity of λ -containing immunoglobulins²³, suggesting similar possibilities for the products of rearranged γ genes. Junction region variations are actually greater among the three rearranged γ -cDNA clones than has so far been observed in λ -chain genes and may in part be due to the involvement of D segments.

In speculating about the possible functions of the γ -gene product, we assume that: (1) the diversity at the V-J junction regions is clonal in nature and (2) the rearranged γ gene is expressed in CTLs but not ordinarily in T-helper cells. As cytotoxic and helper T cells are restricted usually in their recognition of antigen by different major histocompatibility complex (MHC) elements (CTLs by class I and T helpers by class II molecules, reviewed in ref. 24) it is possible that the product of the γ gene is (or is part of) a second receptor, concerned in CTL clones with restriction by MHC class I molecules. In suggesting this possibility, however, we do not wish to imply that the $\alpha\beta$ heterodimer has no relevance for recognition of MHC-restricting elements.

The hypothesis that T cells have two receptors, one for antigen and the other for MHC molecules, together with the contrasting one-receptor hypothesis, has been debated extensively. Although some experimental data seem to eliminate some versions of the two-receptor hypothesis²⁵, various other versions have not been eliminated. The properties of the γ gene force us to consider seriously two-receptor models where we visualize

Fig. 4 Comparison of the nucleotide and deduced amino acid sequences of three γ -gene cDNA clones. The two cDNA clones pHDS4 (described previously as pHDS 4/203, ref. 10) and pHDS34 were isolated from the BALB.B CTL clone 2C. The cDNA clone DFL12 was isolated from the DBA/2 CTL clone DFL12 (ref. 19). The predicted amino-acid sequence of cDNA clone pHDS4 is shown above the nucleotide sequence. DFL12 amino-acid residue positions that differ from pHDS4 are shown below the nucleotide sequence. Numbers on the right indicate nucleotide positions. The V, J, C, transmembrane (TM) and cytoplasmic (CY) regions are indicated by the horizontal arrows. Nucleotides in pHDS34 or DFL12 that are identical with those in pHDS4 are indicated by —. Missing nucleotides are indicated by [].

Methods. A subtraction library of cDNA clones from 2C mRNA was constructed in the vector pBR322 (ref. 10). A library of cDNA clones from DFL12 mRNA was constructed in the pCD vector system by the method of Okayama and Berg³⁵. Nucleotide sequences were determined by the method of Maxam and Gilbert³⁶ following the restriction map and strategy described previously¹⁰.

pHDS4 DFL12	ACAGATCATG ACCAACCTG GGGGAACCA CTGACTGCAT TTTCTGAGA CTATAGCACA GTCCCACTCT CCAGCTGGTG ACCTGAATTT CCAGCTGCA	100
pHDS4 DFL12	GAGCACTTCC TGCTCCCTG TGAAGCAGTC CAACCTTGGG Met Leu Leu Leu Arg Trp Phe Thr Ser Cys Cys Leu Trp Val Phe Gly ATC CTC TGC TGC CTC TGG GTT TTT GGG	188
pHDS4 DFL12	Leu Gly Gln Cys Leu Glu Gln Thr Glu TAA Ser Val Thr Arg Glu Thr Asp Glu Asn Val Gln Ile Ser Cys Ile Val Thr Leu Pro CTT GGG CAG CTG GAG CAA ACT GAA TTA TCG GTC ACC AGA GAG ACA TTT TTT GAG TAT CTA ATA TAT GTC GCA ACA AAC TAC	272
pHDS4 pHDS34 DFL12	Tyr Phe Ser Asn Thr Ala Ile His Trp Arg Gln Lys Thr Asn Gln Phe Glu Tyr Thr Ile Tyr Val Ala Thr Asn Tyr TAT TTC TCC AAC ACA GCT ATA CAT CAT TGG TAC CCG CAA AAA ACA AAT CAA GAG TTT GAG TAT CTA ATA TAT GTC GCA ACA AAC TAC	356
pHDS4 pHDS34 DFL12	Asn Gln Arg Pro Leu Gly GGG Lys Arg His Lys Lys Ile Glu GCA Ser Lys Asp Phe Lys Ser Ser Thr Thr Ser Thr Glu Ile Asn AAT CAA CGA CCC TTA GGA GGG Lys Arg CAC AAA AAA ATT GAA GCA AGT AAA GAT TTT AAA AGT TCT ACC TCA ACC TTG GAA ATA Asn	440
pHDS4 pHDS34 DFL12	TAC Leu Lys Lys Glu Asp Glu Ala Thr Tyr Tyr Cys Ala Val Trp Met Arg Tyr Ser Ser Gly Phe His Lys Val Phe Ala Glu TTC TGG AAG AAA GAA GAT GAG CCC ACT TAC TAC TGT CAA AAA ACA AAT CAA GAG TTT GAG TAT CTA ATA TAT GTC GCA ACA AAC TAC	524
pHDS4 pHDS34 DFL12	Gly Thr Lys Leu Ile Val Ile Ser Ser Asp Asp Arg Leu Asp Ala Asp Ile Ser Pro Lys Pro Thr Ile Phe Leu Pro Ser Val GGA ACA AAG CTC ATA GTA ATT CCC TCC GAC AAA AGG CTT GAT GCA GAC ATT TCC CCC AAG CCC ACT ACT TTT TTT CTT TCT GTT	608
pHDS4 pHDS34 DFL12	Ala Glu Thr Gln Leu His Lys Thr Gly Thr Tyr Leu Cys Leu Leu Glu Lys Phe Phe Pro Asp Val Ile Arg Val Tyr Trp Lys GCT GAA ACA AAT CTC CAT AAG ACT GGG ACA TAC CTT TGT CTC CTT GAA AAG TTC TTT CCC GAT GTC ATA AGG GTG TAT TGG AAA	692
pHDS4 pHDS34 DFL12	Glu Lys Asn Gly Asn Thr Ile Leu Asp Ser Gln Glu Gly Asp Thr Thr Lys Thr Lys Gly Ala Thr Met Lys Phe Ser Trp Leu GAA AAG AAT GGC AAT ACT ATC CTC CAG GAA GGG GAT ACG CTR AAG ACT AAG GGC ACA TAC ATG AAG TTT AGC TGG CTT	776
pHDS4 pHDS34 DFL12	Thr Val Pro Glu Arg Ala Met Gly Lys Glu His Ser Cys Ile Val Lys His Glu Asn Asn Lys Gly Gly Ala Asp Gln Glu Ile ACT GTG CCT GAA AGG GCA ATG GGG AAA AAA GAG CAC AGT TGT ATT GTT GTC AAA CAT GAG AAC AAC AAA GGA GGA GCA GAT CAA GAG ATT	860
pHDS4 pHDS34 DFL12	Phe Phe Pro Ser Ile Lys Lys Val Ala Thr Thr Cys Trp Gln Asp Lys Asn Asp Val Leu Gln Phe Gln Phe Thr Ser Thr Ser TTC TTC CTT TCA ATA AAG AAA GTT GCT GCT ACT ACT TGC TGG CAA GAT AAA AAT GAT GTG CTG CAG TTC CAG TTC ACC ACC TCT	944
pHDS4 pHDS34 DFL12	Ala Tyr Thr Thr Tyr Leu Leu Leu Leu Leu Lys Ser Val Ile Tyr Leu Ala Ile Ser Phe Ser Leu Leu Arg Arg Thr Ser GCC TAC TAC ACC TAC CTC CTC CTC CTC CTC CTC CTC AGT GTG ATC TAC TTG GCC ATC ATC AGC TTC TCT CTT CTT AGA AGA ACA TCT	1028
pHDS4 pHDS34 DFL12	Val Cys Gly Asn Glu Lys Lys Ser TAAAGAAAC AGTGGTGGTA CAGCAAGTCA GCTGGATTTC ATCTCACTG CCATAAAGGT GCCTTAAGGG GTC TGT GGG AAT GAG AAG AAG TCC	1122
pHDS4 pHDS34 DFL12	GGAAACAGAT GCCTTCTCT GTTGGCTTTC ACCTCTATAA AGTCCCTCAC TCATGTTGCA TAAACATTTT CTGAACCTTT GTATGCAATT TCAGCAACTT	1222
pHDS4 pHDS34 DFL12	TTTAAAC TGAACCTACCTT CTCTCTGATT CCATCCCTAC CAGAAGTCCC CTCCCTCCAGA AGCCTGAAAC ATTAAATTC TAGTACCAT AGCTACAGC	1322
pHDS4 pHDS34 DFL12	TTTACCAT GGCCTTGTGT GCTCTTGAAC CAGCTAATC CATGAAGACC CCCACTCTAG ACAACCTGAA GAGCCTTCTT TACTGTGTTA TTCTGTGCTT	1422
pHDS4 pHDS34 DFL12	TGATTTTCAG ATATATGACA ATAAATTTT TAAAAATTA GAAAAA	1468

that one receptor is the $\alpha\beta$ heterodimer recognized already at the protein level and the second receptor includes the γ -gene product. Whether the latter is a homodimer, a heterodimer or a multimer composed of the product of the γ gene and the product(s) of another gene(s), which may or may not be rearranged in T cells, remains unknown.

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Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes

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We have deduced the entire 1,370-amino-acid sequence of the human insulin receptor precursor from a single complementary DNA clone. The precursor starts with a 27-amino-acid signal sequence, followed by the receptor α -subunit, a precursor processing enzyme cleavage site, then the β -subunit containing a single 23-amino-acid transmembrane sequence. There are sequence homologies to human epidermal growth factor receptor and the members of the src family of oncogene products.

THE rapid metabolic effects of insulin and its long-term growth promoting actions are initiated by its interaction with specific, high-affinity cell-surface receptors¹⁻³. The insulin receptor (apparent relative molecular mass (M_r) 350,000–400,000) is an integral membrane glycoprotein composed of two α -subunits (apparent M_r 120,000–130,000) and two β -subunits (apparent M_r 90,000), linked by disulphide bonds⁴⁻⁸. Photoaffinity labelling of the receptor^{9,10} as well as affinity crosslinking^{7,11} have shown that the α -subunit is labelled predominantly by radioactive insulin derivatives. In intact cells, insulin stimulates the phosphorylation of the β -subunit on serine and tyrosine residues, a phenomenon first observed in rat hepatoma cells and IM9 lymphoblasts^{12,13} and then extended to a variety of cell types¹⁴⁻¹⁶. The insulin receptor exhibits insulin-dependent tyrosine kinase activity¹⁴⁻²² that co-purifies with the insulin-binding activity to homogeneity *in vitro*^{19,20,23}. The protein kinase activity of the insulin receptor catalyses phosphorylation of both the β -subunit and exogenous peptides and proteins^{14,21,24-26}. Studies of model peptide substrates indicate that the specificity of the insulin-dependent protein kinase is similar to that of the epidermal growth factor (EGF) receptor kinase and the src family of tyrosine-specific protein kinases^{24,25}. In addition to being the principal substrate for autophosphorylation, the β -subunit has an ATP-binding site^{21,22}, and hence probably contains the protein kinase catalytic domain. Thus, the functional cell-surface insulin receptor possesses both insulin-binding and tyrosine-specific protein kinase activities.

The α - and β -subunits of the heterotetrameric insulin receptor derive from a single glycosylated polypeptide precursor of $M_r \sim 190,000$ (refs 27–29). Disulphide bridges linking the α - and β -subunit regions probably begin to form as the protein folds; after being transported from the endoplasmic reticulum to the Golgi apparatus, the precursor is further glycosylated and then cleaved, followed by its transport to the plasma membrane³⁰. As both subunits of the insulin receptor are glycosylated and the β -subunit contains the ATP-binding site involved in the protein kinase activity, it is probable that portions of both subunits are exposed on the cell surface and that the β -subunit spans the membrane bilayer. The structure of insulin receptor seems to be highly conserved in vertebrate and invertebrate cells^{31,32}. Although similar in structure to the insulin-like growth factor (IGF)-1 receptor³³, the insulin receptor differs from the EGF receptor, which functions in the plasma membrane as a single polypeptide chain.

There is interest in the relationship of the insulin receptor to other growth factor receptors (such as the EGF receptor) possessing both ligand-binding and ligand-dependent tyrosine-specific protein kinase activities. The recently reported com-

plete amino-acid sequence of the human EGF receptor^{34,35} shows that its transmembrane and cytoplasmic domains are homologous to the v-erb-B oncogene product and that these domains represent the corresponding cellular proto-oncogene.

We report here the complete amino-acid sequence of the human insulin receptor precursor deduced from human placental cDNA clones, revealing various structural features of the mature receptor and showing similarities to the EGF receptor and products of the src family of oncogenes.

N-terminal amino-acid sequences

Insulin receptor was purified from human placental membrane preparations (Fig. 1 and ref. 23). Following insulin affinity chromatography, preparative amounts of receptor subunits were resolved by acrylamide gel electrophoresis and were then isolated by electroelution of gel slices (Fig. 1). Amino-terminal sequences were obtained for both α - and β -subunits (Fig. 1b). Residues in the amino-terminal sequences that could not be identified directly were guessed on the basis that they would be amino acids destroyed extensively or otherwise difficult to detect during protein sequencing.

This partially predicted sequence information (Fig. 1b) was used to design single long synthetic DNA probes³⁶ for hybridization screening of a DNA library to identify human insulin receptor cDNA clones. Double-stranded probes were generated by synthesizing short overlapping oligonucleotides for both α - and β -sequences³⁷. Subsequent phosphorylation with high specific activity [γ -³²P]+ATP and ligation of oligonucleotides (see Fig. 1 legend) resulted in radioactive double-stranded 63-(α -subunit) and 57-(β -subunit) base-pair (bp) long probes. Total poly(A)-containing RNA from human term placenta was used to construct a cDNA library ($\sim 1.5 \times 10^6$ clones) with the λ gt10 vector system³⁸ (see Fig. 2 legend).

Insulin receptor cDNA

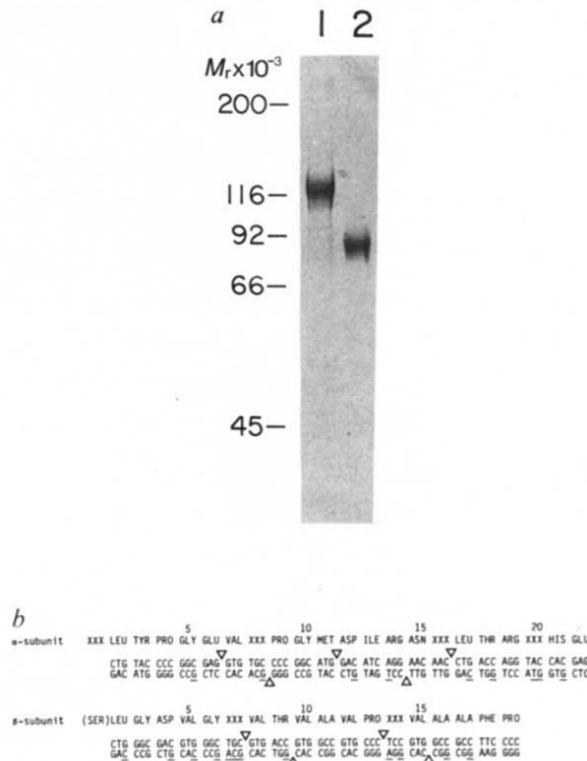
Initial screening with the α -subunit probe detected 15 hybridization-positive recombinant phage. Characterization of purified phage DNAs by *Eco*RI restriction analysis followed by Southern blot hybridization³⁹ shows that each clone contains two *Eco*RI fragments, one of which hybridizes with the synthetic α -subunit probe. Only one phage (λ HIR-P12) contains a second *Eco*RI fragment hybridizing with the β -subunit probe. The entire cDNA insert of phage λ HIR-P12 measures ~ 5 kilobases (kb) (1-kb and 4-kb *Eco*RI fragments, Fig. 2a) and is therefore large enough to code for the entire human insulin receptor precursor, estimated to be of $M_r \sim 190,000$ including carbohydrate side chains²⁹.

Fig. 1 Insulin receptor purification and protein sequence analysis. **a**, Coomassie blue-stained SDS-polyacrylamide gel of electrophoretically purified α - (lane 1) and β -subunits (lane 2). The positions of the M_r standards (myosin, β -galactosidase, phosphorylase *b*, bovine serum albumin and ovalbumin) are indicated. **b**, Amino-terminal amino-acid sequences of insulin receptor α - and β -subunits and nucleotide sequences of α - and β -subunit probes. Codons for (XXX) positions were chosen on the basis of tentative amino-acid assignments; parentheses indicate uncertainty in assignment; arrows indicate ligation points between independently synthesized oligonucleotides; underlined nucleotides represent mismatches with the natural insulin receptor cDNA complement.

Methods. The insulin receptor was purified from human placental membrane preparations by chromatography on wheat-germ agglutinin agarose and insulin agarose as described previously, except that phenylmethylsulphonyl fluoride (PMSF) was used to inhibit proteolysis; elution from wheat-germ agglutinin was performed at 4 °C and the protein eluted from the insulin column with 0.5% SDS. Chromatography on hydroxylapatite in SDS⁶¹ was used to concentrate the partially purified receptor as follows: the insulin agarose eluate was diluted to <0.2% SDS and made up to 0.01 M in sodium phosphate pH 6.4 and 1 mM in dithiothreitol and passed over a 3-ml column of hydroxylapatite at 37 °C; bound protein was eluted with 0.6 M sodium phosphate pH 6.4, 1 mM dithiothreitol, 0.1% SDS. Purification of the subunits was obtained by preparative polyacrylamide gel electrophoresis after dialysis against 0.1% SDS. Samples were electrophoresed on 7% gels with 1 mM sodium thioglycolate in the upper reservoir buffer and bands were excised from the Coomassie blue-stained gel and electroeluted as described previously⁶². Quantitation of the α - and β -subunits was based on a determination of the staining intensity of individual bands with Coomassie blue after polyacrylamide gel electrophoresis. Staining was determined by laser densitometry (Model 2202 Ultrascan, LKB) and the receptor bands compared with known amounts of standard proteins run on the same gel. But the protein

sequence analysis results suggest that our estimate of the amount of receptor is low, particularly for the β -subunit. Purified protein was applied to the vapour phase protein sequencer (Model 470A, Applied Biosystems) described by Hewick *et al.*⁶³ and the amino-acid derivatives released determined by reversed-phase HPLC on a Microsorb C8 column (4.6 × 250 mm, Rainin) using an aqueous phosphate buffer with acetonitrile as the organic solvent. For the α -subunit, 65 pmol of sequence was obtained from an estimated 130 pmol of protein, whereas 400 pmol of sequence was obtained from an estimated 300 pmol of the β -subunit. Double-stranded hybridization probes were prepared using an automatic DNA synthesizer (Biosearch). Short overlapping oligonucleotides were synthesized and purified by acrylamide gel electrophoresis; ~10 pmol of each oligonucleotide were phosphorylated in separate reactions using threefold excess of [γ -³²P]+ATP (Amersham) and T4 polynucleotide kinase. Full-length probes were prepared by combining the 5'-end-labelled oligonucleotides and ligation with 2 U of T4 DNA ligase at 20 °C for 2 h. Analytical examination of the ligation result demonstrated that about 69–80% of the DNA had been ligated to probe monomer size. The entire ligation mixture was boiled to separate DNA strands and used for hybridization as described previously⁴¹.

Nucleotide sequence analysis confirms that the 1-kb *Eco*RI fragment has an open reading frame coding for the amino-terminal sequence of the insulin receptor α -subunit. We find that the leucine residue at position two of the mature α -subunit is preceded by a histidine and identify a potential initiation ATG codon 27 amino acids upstream from His 1, flanked by nucleotides matching Kozak's⁴⁰ criteria for a translation initiation site. The amino-acid residues between the ATG codon and the NH₂-terminus of the mature protein are highly hydrophobic and probably represent the signal peptide sequence necessary for transport of the nascent insulin receptor precursor polypeptide into the lumen of the endoplasmic reticulum. Even though the remaining nucleotide sequence upstream from Met 27 does not contain any in-frame stop codons, the presence of an adjacent suitable signal sequence led us to propose that the ATG at position -27 is used for translation initiation. This assignment predicts the orientation of the α - and β -subunit sequences in the insulin receptor precursor: the α -subunit of the receptor is expected to be upstream of the β -subunit NH₂ terminus and in their common precursor. This prediction was confirmed by determination of the complete 5,181-bp-long nucleotide sequence of the λ HIR-P12 cDNA insert (Fig. 2b). The longest open reading frame starting with methionine in this sequence codes for 1,370 amino acids, including the 27-residue signal peptide. The coding sequence is preceded by 50 nucleotides of 5'-untranslated sequence and is followed by a signal for translational termination (TAA) and 1,018 nucleotides of 3'-untranslated sequence. It is not certain that the A-stretch at the 3' end of our sequence represents part of a poly(A) tail or an



internal sequence in a larger 3'-untranslated region, as this A-stretch is preceded by an imperfect polyadenylation signal (AATATA). To resolve this question, we selected three additional independent cDNAs representing 3'-untranslated sequences from the placental library and compared them by restriction analysis with λ HIR-P12 *Eco*RI fragments. Each of them seems to end with the same 3'-terminal sequence and matched λ HIR-P12 restriction patterns, although none extends as far upstream (data not shown).

Comparison of final cDNA-derived nucleotide sequences with our synthetic probes reveals that the α -subunit N-terminal probe contains nine mismatches (86% match) with the longest stretch of perfect match being 11 bp; the 57-bp, β -subunit N-terminal probe contains 12 mismatches (79% match) and a 12-bp maximum uninterrupted match. We identify no false positives, again confirming the effectiveness of the long synthetic probe screening approach^{41–43}.

Based on our cDNA sequence, we calculated the M_r to be 155,000 for the pre-insulin-receptor precursor and 152,000 for the mature precursor. A tetrapeptide (Arg-Lys-Arg-Arg) at position 720 of the insulin receptor precursor sequence directly precedes the β -subunit N-terminal sequence (Fig. 2b) and probably represents the cleavage site for the receptor precursor processing enzyme. Omitting this peptidase recognition sequence, the final unmodified subunit M_r s are predicted to be 82,400 (α) and 69,700 (β).

The 719-residue-long α -subunit sequence (Fig. 2b) is largely hydrophilic (Fig. 2c) with a few short hydrophobic stretches, none long enough to qualify as potential membrane anchor

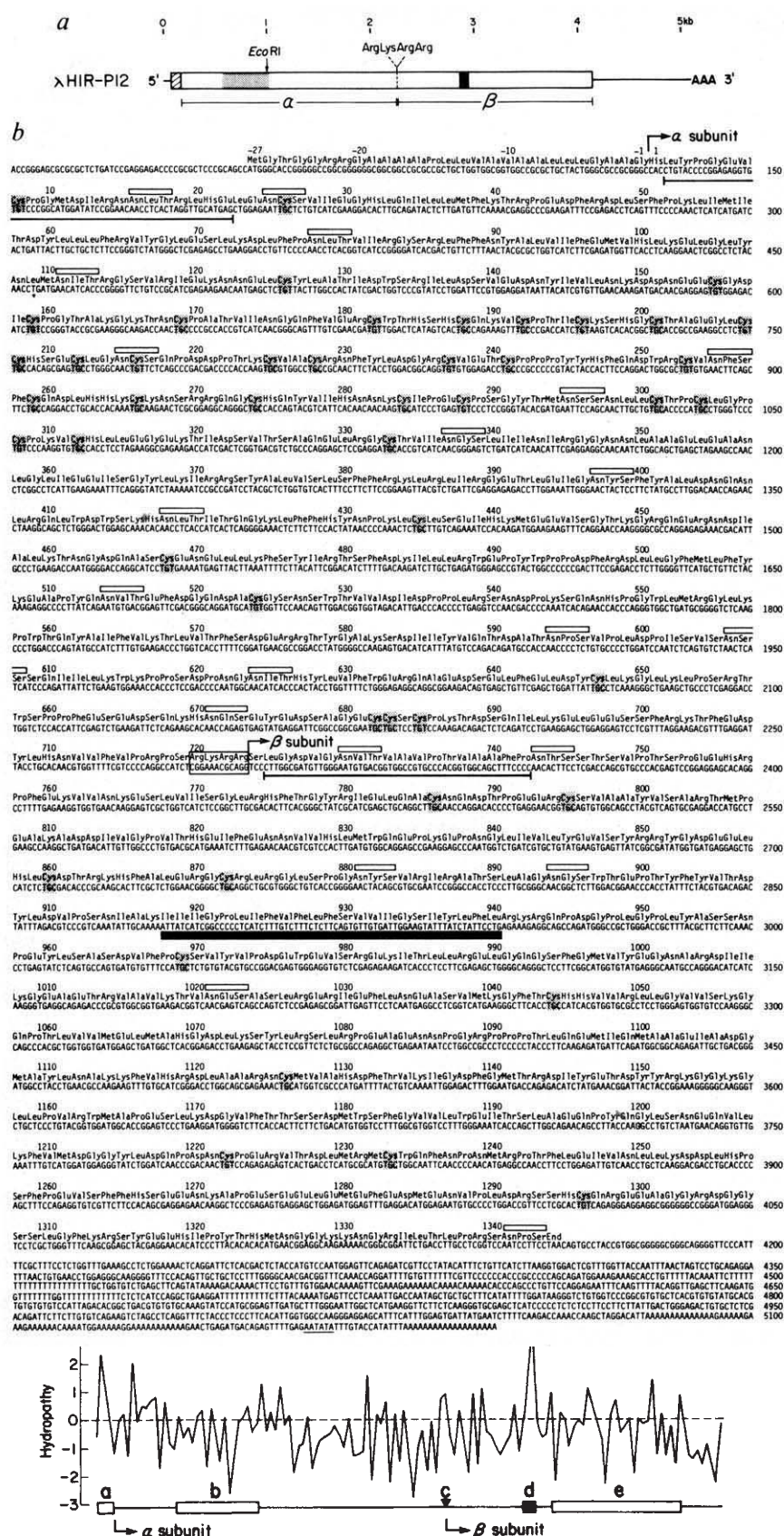


Fig. 2 Insulin receptor precursor cDNA. **a**, Schematic diagram of clone λHIR-PI2. Indicated are the single internal *EcoRI* restriction site, untranslated sequences (—), translated sequences (boxed), signal sequence (hatched), cysteine-rich region (shaded), putative precursor processing site and the transmembrane domain (black bar). **b**, Nucleotide sequence of insulin receptor precursor cDNA and deduced amino-acid sequence. Amino acids numbered starting at His 1 of the proreceptor sequence are preceded by a 27-residue signal sequence. Experimentally determined amino-terminal sequences of insulin receptor α - and β -subunits (Fig. 1) are underlined; black bar, putative transmembrane sequence; open bars, consensus sequences for N-linked glycosylation; shading, cysteine residues; boxes, precursor processing site. **c**, Hydropathy analysis. The 1,370-amino-acid-long pre-insulin-receptor-precursor sequence was scanned using the computer program of Kyte and Doolittle⁶⁶. Landmarks in the sequence are indicated schematically: signal sequence (a), cysteine-rich region (b), precursor processing site (c), transmembrane sequence (d) and tyrosine kinase domain (e). Hydropobicity results in positive and hydrophilicity in negative values (see ref. 66). **Methods:** **a**, Total poly(A)-containing RNA was isolated from frozen term placenta. A clone library (1.5×10^6 plaque-forming units with cDNA (> 500 bp) and the λ gt10 vector system was prepared as described previously^{35,38}. Nucleotide sequence analysis was performed using the enzymatic chain termination method in conjunction with M13-derived vectors^{64,65}.

sequences. Fifteen consensus sequences for asparagine-linked glycosylation (Asn $\times$ Ser/Thr) are distributed evenly over the 719-residue-long α -subunit region, which is also characterized by an unusually large number (37) of cysteine residues, 26 of which are concentrated between residues 155 and 312 (Fig. 2b) in a rather hydrophilic domain (Fig. 2c). Although in most cases

there is no direct evidence as to which of the potential asparagine-linked carbohydrate attachment sites are in fact glycosylated, our failure to detect the asparagines at positions 16 and 7 of the α - and β -subunits, respectively, during protein sequencing strongly suggests that these two sites are glycosylated.

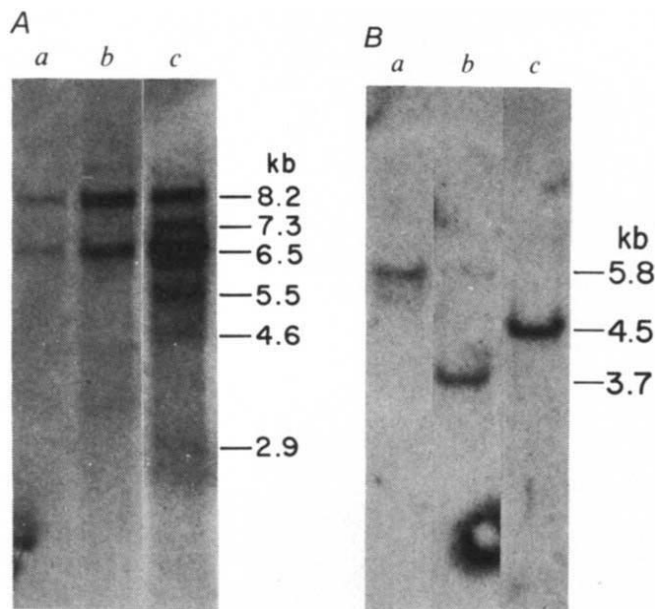


Fig. 3 Hybridization analyses. **A**, Northern blot analysis⁴⁴ of poly(A)⁺ RNA (5 µg) isolated from mouse 3T3-L1 fibroblasts before (a) and after differentiation into adipocytes⁴⁶ (b) and human term placenta tissue (c) after separation on a formaldehyde-containing 1% agarose gel and transfer to nitrocellulose. Rat ribosomal RNA was used as a size marker. The filter was hybridized with radiolabelled insulin receptor cDNA fragments⁶⁷ (1,010 bp and 4,169 bp *Eco*RI). Exposure was for 3 days at -60 °C using an intensifying screen (Cronex Lightning Plus). Additional Northern blot experiments with placenta and IM-9 RNAs were performed as described in the text. The following cDNA fragments were used in parallel experiments: *Eco*RI 1/*Eco*RI 1,011; *Eco*RI 1,012/*Xho*I 2,904; *Stu*I 3,234/*Stu*I 3,728; *Stu*I 2,399/*Xho*I 3,080; *Pst* I 4,341/*Eco*RI 5,169. **B**, Genomic Southern blot analysis. High M_r DNA (10 µg per lane) isolated from placental nuclei was digested with excess amounts of *Eco*RI (a), *Hinc*II (b) and *Pst*I (c), separated on a 1% agarose gel and analysed by Southern blot hybridization³⁹. The radiolabelled probe used in high stringency conditions consisted of a 857-bp 3'-terminal *Pst*I/*Eco*RI fragment. λ wt phage DNA digested with *Eco*RI and *Hind*III was used as size marker.

The 620-amino-acid-long β-subunit sequence contains only nine cysteine residues (1.5%) and can be divided into three domains. The amino-terminal 194-residue-long domain contains four potential asparagine-linked glycosylation sites and four cysteine residues. An adjacent stretch of 23–26 highly hydrophobic amino acids (915 or 918–940) probably represents the single transmembrane domain that anchors the insulin receptor in the membrane. The transmembrane sequence is flanked at its C-terminal end by three basic amino acids (Arg-Lys-Arg; Fig. 2b), which are part of the 403-residue-long carboxy-terminal domain containing two potential N-linked glycosylation sites and a typical number of cysteine residues.

Multiple related mRNAs

Northern blot hybridization⁴⁴ analysis using poly(A)⁺ RNA from term placenta (Fig. 3A, c), as well as fetal placenta (20 week) and a human lymphoblast cell line (IM-9) (data not shown), yields a complex pattern of five common hybridization bands 8.2, 7.3, 6.5, 5.5 and 4.6 kb in length. A faint 2.9-kb band also occurs, but is present only in term placenta and IM-9 RNAs. The hybridization signal intensity for the bands is different for each band, indicating that either variable amounts of different length messenger RNAs are synthesized from the same gene, or that different but related genes are transcribed to yield multiply-sized mRNAs. To further investigate the identity of mRNAs from placenta and IM-9 cells which hybridize with our 5.2-kb

insulin receptor cDNA probe, we used a variety of cDNA probe fragments as hybridization probes in Northern blot hybridization experiments (see Fig. 3A legend). In all cases, at least all of the four largest transcripts (8.2, 7.3, 6.5 and 5.5 kb) are detected (data not shown).

After treatment with dexamethasone and isobutyl methylxanthine, mouse 3T3-L1 fibroblasts differentiate into adipocytes in a process which leads to a 10- to 20-fold increase in the number of insulin receptor molecules on their surfaces^{45,46}. Northern blot analysis with poly(A)-containing mRNA from these cells before and after differentiation reveals that the increase in receptor molecules parallels an ~10-fold increase in two insulin receptor mRNAs (Fig. 3A). Unlike the complex transcription pattern in placenta (Fig. 3A, c), the 3T3-L1 cells synthesized only comparable amounts of a 6.5- and an 8.2-kb mRNA, both before and after induction. These experiments strongly suggest that the major transcripts of 6.5 and 8.2 kb represent insulin receptor mRNAs. The mRNAs may differ in the lengths of their 3'- or 5'-untranslated sequences, as described for other gene systems^{47,48} or alternatively these mRNAs could be generated by variable splicing of a primary transcript from the same gene.

One receptor gene

To determine the number of insulin receptor genes present in the human genome, Southern blot analyses³⁹ were performed using an 857-bp *Pst*I/*Eco*RI cDNA fragment derived from the 3'-most terminal untranslated sequence as a hybridization probe. These sequences were chosen because they are the most rapidly divergent sequences in gene families and would therefore avoid detection of closely related genes. Figure 3B demonstrates that this probe hybridizes with single restriction fragments from high M_r nuclear DNA, consistent with the presence of only one insulin receptor gene in the haploid human genome.

Discussion

Here we present the primary structure of the human insulin receptor precursor, deduced from the sequence of a single cDNA clone. The amino-acid sequence contains the amino-terminal sequences of the α- and β-subunits, determined by sequencing purified insulin receptor subunit protein. The sequence further establishes that the organization of the α- and β-subunit sequences in the receptor precursor is α followed by β. The insulin receptor precursor has a signal peptide sequence at its amino terminus immediately adjacent to the amino terminus of the α-subunit sequence, which is removed from the precursor during its biosynthesis.

The predicted M_r of the 1,343-residue-long proreceptor polypeptide backbone is 152,000, after removal of the signal peptide. Pulse-chase studies in the presence of tunicamycin have suggested that the non-glycosylated receptor precursor has an apparent M_r of 180,000 (ref. 29). The large precursor may have an anomalous electrophoretic mobility; SDS-polyacrylamide gel electrophoresis does lead to an overestimate of the true M_r of the α-subunit⁴⁹. Both subunits are separated by the tetrapeptide sequence Arg-Lys-Arg-Arg, which probably represents the recognition sequence for the precursor processing enzyme. Enzymes that recognize paired basic sequences often cleave multiply in such a sequence, resulting in the loss of amino acids from the processed protein products. Therefore, we omitted these residues in calculating the M_r of the unglycosylated mature α- and β-subunits, 82,500 and 69,700 respectively, compared with 120,000–130,000 (α) and 90,000 (β) for the modified subunits. The difference in M_r of the unmodified proreceptor and the mature glycosylated molecule suggests that most, if not all, of the potential N-linked glycosylation sites are glycosylated.

A proposed schematic arrangement of the insulin receptor subunits is shown in Fig. 4. As the α-subunit lacks any hydrophobic stretches long enough to serve as membrane anchor sequences and contains 15 of the 21 potential N-glycosylation sites of the receptor, this subunit is probably exclusively in the

extracellular domain and is held in place by disulphide links to the β -subunit. The presence of a clearly recognizable 23-amino-acid-long transmembrane segment in the β -subunit (residues 918–940) fairly analogous to the transmembrane regions of the EGF and low-density lipoprotein (LDL) receptors^{35,50}, indicates that the amino terminus of the subunit is exposed on the cell surface with the carboxy-terminal region from residues 941–1343 facing the cytoplasmic side of the plasma membrane. Such an orientation would place four of the six *N*-glycosylation sites of the β -subunit outside the cell.

The predominant labelling of the α -subunit by radioactive insulin derivatives in affinity crosslinking and photoaffinity labelling experiments has already suggested that insulin interacts with its receptor by binding to the α -subunit^{9–11}, although the β -subunit amino terminus may also be involved. The α -subunit contains a region very rich in cysteine residues (residues 159–312), similar to that present in the EGF receptor³⁵ as well as in the ligand binding domain of the LDL receptor⁵⁰. The extracellular domains of both the EGF receptor and LDL receptor, however, contain two such cysteine-rich clusters. Each α -subunit of the heterotetrameric insulin receptor may contribute a Cys-rich region to form an analogous functional domain.

The structure of the human insulin receptor reveals striking similarities to the human EGF receptor in its overall organization and primary sequence³⁵. The first 444 residues of the mature insulin receptor α -subunit are 27% homologous to the first 461 residues of the EGF receptor; 20% of these homologous residues are cysteines and are part of the cysteine-rich domains of both receptors (Figs 4, 5). As the intracellular domain of the EGF receptor seems to have been generated by a sequence duplication³⁵, this domain contains two cysteine-rich regions, both homologous to the membrane-distal portion of the insulin receptor. Both the insulin receptor and the EGF receptor span the membrane once with a hydrophobic 23-amino-acid sequence. Interestingly, the sequences on either side of the membrane are conserved between these two receptors and the LDL receptor. The insulin and EGF receptor each have three basic amino acids on the cytoplasmic side of the membrane (Arg-Lys-Arg and Arg-Arg-Arg, respectively)³⁵, whereas the LDL receptor contains the basic sequence⁵⁰ Lys-Asn-Trp-Arg-Leu-Lys. On the extracellular side of the membrane, each of these receptors contains a Pro-Ser sequence flanking the membrane anchor sequence, as well as other conserved residues. Basic sequences also occur at the cytoplasmic domain membrane junctions of other plasma membrane proteins^{51–55}. In addition to their potential interaction with the head groups of negatively-charged phospholipids, the density of charged residues probably stops further transfer of intracellular protein sequences across the membrane during the co-translational translocation of the nascent polypeptides⁵⁶.

A comparison of the insulin receptor β -subunit cytoplasmic domain with analogous portions of members of the *src* family of tyrosine-specific protein kinases (Fig. 5b) begins at the ATP-binding sites, located precisely 49 amino-acid residues from the membrane in both the insulin and EGF receptors, as well as in the deduced sequence of the product of the *v-fms* oncogene⁵⁷. Large clusters of homology occur throughout this region, including a lysine residue essential for kinase activity 25 amino acids from the ATP-binding site⁵⁸ and the tyrosine residue autophosphorylated in *v-src*⁵⁹ (position 1,150 in human insulin receptor). Autophosphorylation of the EGF receptor occurs downstream from the region compared in Fig. 5b⁶⁰. It is not yet known which tyrosine residue is phosphorylated in the insulin receptor; however, a sequence homologous to that surrounding the major phosphorylated tyrosine residue of the EGF receptor (1,173)⁶⁰ flanks the tyrosine at position 960 of the insulin receptor.

These structural homologies demonstrate that the insulin receptor is a member of the *src* family of tyrosine-specific protein kinases. The insulin receptor is related equally to each of the *src* family members, which suggests either that it differs from the EGF receptor and is not a cellular proto-oncogene, or that

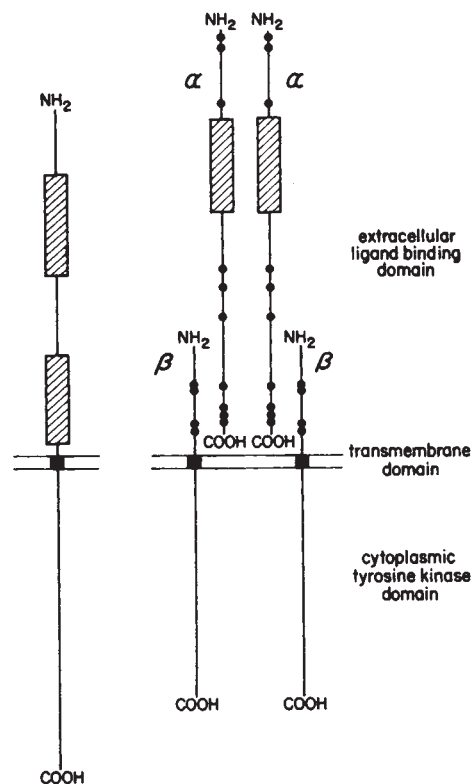


Fig. 4 Schematic comparison of insulin and EGF receptors. Regions of high Cys-residue concentration are shown as hatched boxes, transmembrane domains as black boxes and single cysteine residues, possibly involved in formation of the α_2 - β_2 insulin receptor complex, as black circles.

its oncogene counterpart has not yet been identified. The cellular events triggered by the binding of ligand to the insulin and EGF receptors are different, even though these receptors share multiple structural and functional features.

Southern hybridization experiments demonstrate the existence of a single insulin receptor gene. The insulin receptor cDNA hybridizes to six mRNAs between 2.9 and 8.2 kb in placenta and IM-9 cells, at least four of which are derived from the same gene. Two mRNAs of 6.5 and 8.2 kb yield the most intense hybridization signals and were both induced ~10-fold on differentiation of mouse 3T3-L1 fibroblasts into adipocytes. We believe that our insulin proreceptor cDNA is derived from one of these two mRNAs, which may differ with respect to the length of their 3'- or 5'-untranslated sequences, as demonstrated in other gene systems. Our experiments further reveal a high degree of homology and length conservation between the mouse insulin receptor mRNAs and their human counterparts. Future experiments will elucidate the significance of this transcriptional complexity and determine the relationship between the insulin receptor gene and the gene for the structurally related IGF-1 receptor³³.

Isolation and characterization of the insulin receptor cDNA will enable the investigation of functional aspects of the receptor in normal cells and in heritable syndromes of insulin resistance. Site-directed modification of this cDNA followed by expression of mutated insulin receptor molecules in mammalian cells will help to elucidate the molecular mechanisms of insulin action.

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Structure of large fragment of *Escherichia coli* DNA polymerase I complexed with dTMP

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The 3.3-Å resolution crystal structure of the large proteolytic fragment of Escherichia coli DNA polymerase I complexed with deoxythymidine monophosphate consists of two domains, the smaller of which binds zinc-deoxythymidine monophosphate. The most striking feature of the larger domain is a deep crevice of the appropriate size and shape for binding double-stranded B-DNA. A flexible subdomain may allow the enzyme to surround completely the DNA substrate, thereby allowing processive nucleotide polymerization without enzyme dissociation.

ERROR-FREE replication of parental DNA by polymerases is essential for the continuity of organisms from one generation to the next. The library of genetic information in the chromosomal DNA must be copied rapidly and accurately into daughter DNA. As the ability to pass on genetic information was presumably required for prebiotic evolution, DNA polymerase activity must have been among the earliest catalytic activities. We report here the first crystal structure of a polymerase, the large proteolytic fragment of *Escherichia coli* DNA polymerase I, which may assist eventually in elucidating both the mechanisms used by the enzyme to enhance the fidelity of template-directed polymerization and the origins of polymerase activity.

The two major roles of DNA polymerase I (Pol I) in *E. coli* are repair of damaged duplex DNA and the processing of Okazaki fragments¹. The molecule is a monomer of relative molecular mass (M_r) 103,000 and it has three enzymatic activities: DNA polymerase, a 3'-5' exonuclease thought to edit out mismatched nucleotides and a 5'-3' exonuclease. Limited proteolysis cleaves the molecule into two fragments^{2,3}; the large C-terminal fragment (the Klenow fragment) has both the DNA polymerase and the 3'-5' exonuclease activities, whereas the smaller amino-terminal fragment has the 5'-3' exonuclease activity. It was previously thought that Pol I was a zinc metallo-enzyme⁴. However, recent work⁵ demonstrates that Pol I is not a zinc enzyme and does not require zinc for any of its enzymatic activities.

Kinetic studies⁶ and the work reported here suggest that the active sites of the DNA polymerase and the error correcting 3'-5' exonuclease activities are to some extent separate on Pol I. The polymerase active site binds double-stranded DNA containing a single-stranded 5' extension and deoxynucleoside triphosphate (dNTP)¹. Although deoxynucleoside monophosphates (dNMP) bind also to Pol I, they do not compete, hence their binding sites are separate^{7,8}. The dNMP seems to bind to the 3'-5' editing exonuclease active site, as dNMP inhibits the 3'-5' exonuclease activity but not the polymerase activity⁶, presumably by product inhibition.

Crystal structure analysis of the Klenow fragment was initiated when the DNA encoding the Klenow fragment was cloned in a high expression vector⁹. The subsequent availability of large quantities of pure protein has facilitated the growth of crystals diffracting to 2.5 Å resolution^{10,11}. We report here the structure of the Klenow fragment complexed with deoxythymidine monophosphate (dTMP) derived by fitting its known¹² amino acid sequence to a 3.3 Å resolution electron density map.

Structure determination

Crystals of the Klenow fragment are tetragonal, space group $P4_3$, $a = b = 102.9$ Å, $c = 85.8$ Å (ref. 10). We collected data on the parent protein crystals and seven potential heavy atom

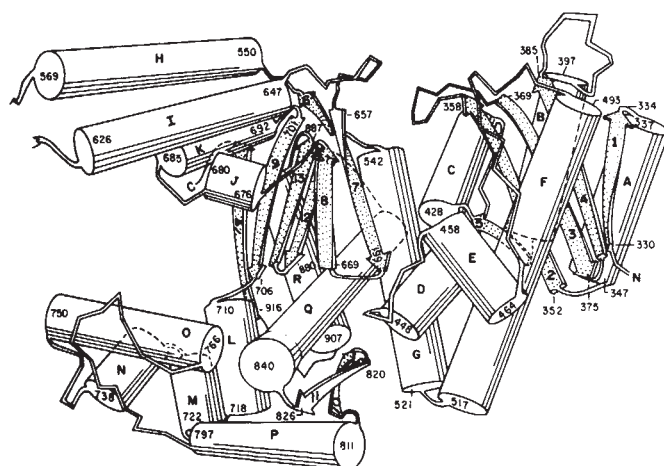


Fig. 1 Schematic drawing of the course of the polypeptide in the Klenow fragment. Regions in β -sheet structure are represented by arrows numbered from the fragment N-terminus and those in α -helix are represented by tubes lettered from the N-terminus. The break indicated between helices I and H is at the position of a partially disordered 50 residues (not included). The division between large and small domains is the loop between helices F and G.

derivatives using the Mark II two-dimensional position sensitive detector¹³. Each complete derivative data set was obtained in only 2 days using one crystal per data set; typically, 80,000 reflections were measured to give 20,000 unique reflections to 2.8 Å resolution. The merging R -factors $= (|I_{i,h} - \langle I_h \rangle|) / \langle I_h \rangle$ (where $I_{i,h}$ = intensity of the i th observation of reflection h) ranged between 0.05 and 0.06 for these data sets. Data from a native protein crystal collected using the two-dimensional detector were compared with data from the diffractometer using the Wyckoff scan algorithm. The R -factor between the two data sets was 0.065 to 3.5 Å resolution.

Four heavy atom derivatives were used in the phasing using the standard method of multiple isomorphous replacement (Table 1). 5-Mercury deoxyuridine monophosphate (5-Hg-dUMP) and $\text{Pt}(\text{NO}_2)_4^{2-}$ were used to 3.3 Å resolution whereas the other derivatives were used only to 3.5 Å ($\text{Pt}(\text{CN})_4^{2-}$) and 4 Å ($\text{Au}(\text{CN})_2^{2-}$) resolutions.

A protein model incorporating the known amino acid sequence¹² was fitted to the electron density map using an Evans and Sutherland colour PS 300 and the computer program FRODO¹⁴ (modified by J. Pflugrath). The protein coordinates have been partially refined at 3.0 Å resolution to a R -factor of 0.31 using Konnert-Hendrickson¹⁵ restrained least-squares refinement. The calculated phases have been combined with the experimental phases¹⁶ and the resulting hybrids used to calculate a map improved in some of the poorly-ordered regions.

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Fig. 2 A model of dTMP fitted into a 3.0 Å resolution difference electron density map calculated using ($F_{\text{dTMP}} - F_{\text{NAT}}$) as coefficients in the Fourier. The F_{dTMP} values were measured from crystals soaked in solutions containing 14 mM dTMP, 20 mM MgSO_4 , 1 mM ZnSO_4 , 70% saturated ammonium sulphate and 66 mM PIPES buffer, pH 7.0; the F_{NAT} values were without the dTMP and MgSO_4 . Phases were calculated from the protein coordinates only. The position of mercury in 5-Hg-dUMP (HG) is near the 5-methyl position of thymine and the zinc (ZN) position is in bonding distance of an oxygen of the 5' phosphate. These metal positions are consistent with the orientation and conformation of dTMP shown. There is additional difference electron density adjacent to the 5' phosphate arising from the binding of another small molecule to the complex.

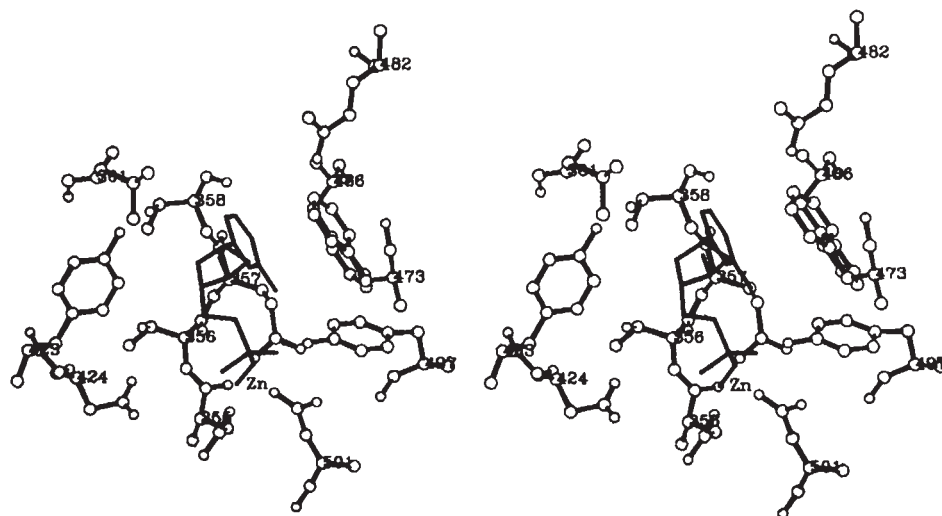
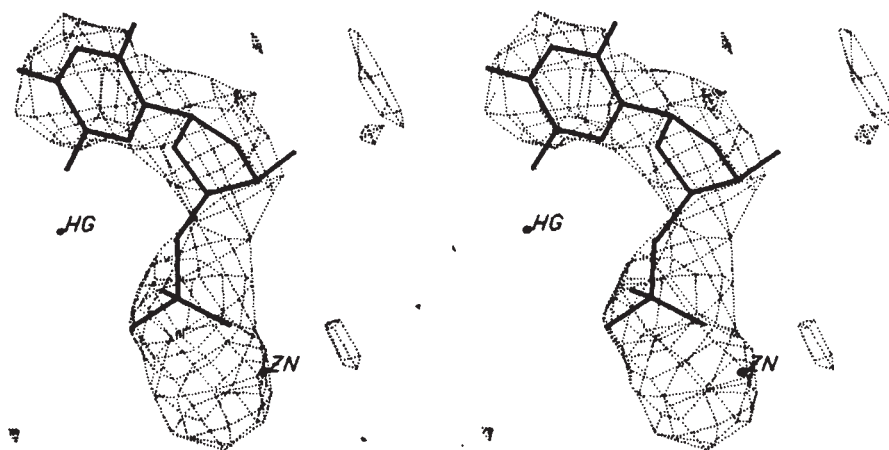


Fig. 3 A stereo drawing of Zn-dTMP and some of the residues in the 3'-5' exonuclease active site with which it interacts. The small molecule bound between the carboxylate of Asp 424 and the 5' phosphate is not shown.

Structure of the Klenow fragment

We find that the fragment is folded into two distinct domains (Fig. 1), the smaller of which is approximately the first 200 residues (324–517 in the Pol I sequence¹², which form a central, mostly parallel β -pleated sheet with α -helices on both sides. This domain has a structure reminiscent of many proteins, for example the nucleotide-binding domains of kinases¹⁷ and dehydrogenases¹⁸. However, there does not seem to be any strong structural homology between these proteins, as superposition of the three antiparallel β -strands 2, 3 and 4 of Klenow fragment on three anti-parallel strands of either β -sheet in yeast hexokinase does not align any other structural features. Deoxynucleoside monophosphate binds to this domain near the carboxyl end of β -strand 2, a position generally similar to the location of the NAD- and ATP-binding regions in dehydrogenases and kinases^{17,18}. However, the orientation and position of the dTMP relative to the β -sheet is different in detail from the cofactor orientations in kinases and dehydrogenases.

The small domain contains a bound metal interacting with both the protein and the 5' phosphate of dTMP. Comparison of crystals soaked in Zn^{2+} , Mn^{2+} , Sm^{3+} or Mg^{2+} with metal-free crystals reveals a tight metal binding site that can bind any of these metals. In the presence of 3 M $(\text{NH}_4)_2\text{SO}_4$, 20 mM MgSO_4 and 1 mM Zn^{2+} , the protein binds a Zn^{2+} atom rather than the Mg^{2+} . However, in 0.2 M MgSO_4 and 2 M Li_2SO_4 , Mg^{2+} binds to the metal site (K. Kornfield, C. Kline, D.L.O. and T.A.S.,

unpublished data). The metal is bound to the protein by the carboxylate groups of Asp 355, Glu 357 and Asp 501. In the dTMP complex, the 5' phosphate provides a fourth ligand to the metal; this metal binding site may account for the earlier conclusion⁴ that Pol I is a zinc metalloprotein.

The 400 amino acids of the larger domain are mainly in α -helical conformation and form a structure containing a very deep cleft similar to the shape of a right hand holding a rod (Fig. 1). A six-stranded anti-parallel β -sheet forms the bottom of the cleft and large protrusions of α -helix on either side form its sides. The cleft is about 20–24 Å wide and 25–35 Å deep with two α -helices, J and K, protruding into it. One side of the cleft is much longer (50 rather than 20 Å) and can be compared with the curled over fingers of a right hand. The other wall is formed primarily by two long α -helices, I and H, projecting from the protein and hanging over the crevice like a thumb.

About 64 amino acid residues are not sufficiently well ordered in this crystal form for an interpretation of their electron density. Of perhaps most functional significance are the 50 residues that lie between the C-terminus of α -helix H (Fig. 1) and the N-terminus of α -helix I that cannot be placed with certainty. Although there is some electron density at a level higher than the general solvent, it is uninterpretable in the present maps. Thus, it seems that a small domain of ~50 residues lying at the tip of the 'thumb-like' protrusion is segmentally disordered or flexible in the crystal. The position of this domain would allow it to close off the fourth side of the cleft.

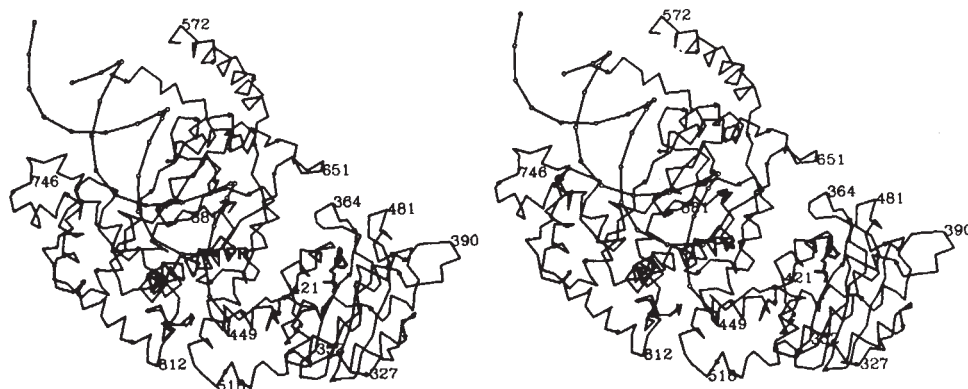


Fig. 4 Stereo drawing of α -carbon backbone of the Klenow fragment including the dTMP and a proposed position for bound duplex DNA. The double-stranded DNA is represented by lines connecting the phosphorus positions, and a possible location of the primer terminus is labelled PR. dTMP is shown with its 5' phosphate bonded to zinc.

Substrate binding

In these crystals, deoxynucleoside monophosphates bind to a single site lying on the small domain^{10,11}. A 3.0 Å-resolution difference electron density map between the enzyme and its complex with dTMP, using phases calculated from the partially refined protein coordinates, is consistent with the conformation of the bound dTMP having approximately the same conformation as in B-DNA (Fig. 2). There are few, if any, significant changes in the protein structure caused by dTMP binding.

The specificity of the dNMP binding site derived previously¹ is consistent with the interactions between dTMP and the protein shown in Fig. 3. First, modification of the 5' phosphate oxygen atoms abolishes deoxynucleotide monophosphate (dNMP) binding¹, implying that all available phosphate oxygen atoms make important interactions. We observe that phosphate oxygen atoms are interacting with: (1) the metal atom; (2) the hydroxyl of Tyr 497 and (3) an unknown small molecule, possibly an additional metal atom, lying between Tyr 497 and Asp 423 (Fig. 2). Second, the 3' OH-group is buried and H-bonded to the backbone amide of Thr 358, which may explain the failure of dideoxynucleoside monophosphates to bind¹. That the 3'-OH is buried and apparently H-bonded is compatible with the NMP-binding site being the exonuclease active site, but is not consistent with it being the binding site for the primer terminus. Third, there are no specific H-bonding interactions between the thymine base and the protein; this is expected because of the ability of the dNMP binding site to bind any of the four bases or many modified bases¹. Instead, the base is bound by several surrounding hydrophobic protein side chains, most notably Leu 361, whose side chain interacts with one side of the base, and Phe 473, interacting on the other side. In summary, dTMP is bound to the enzyme with its phosphate and ribose moieties nearly buried whereas the Watson-Crick H-bonding groups on

the base are free and pointing towards the solution and not the interior of the molecule.

Comparison of the dNMP binding site of Pol I with the active site of staphylococcal nuclease¹⁹ shows some striking similarities and important differences. The essential Ca^{2+} in staphylococcal nuclease is bound to the protein in part by the carboxylate groups of two Asp residues and to an inhibitor nucleotide through an oxygen of its 5' phosphate. Further, a glutamic acid carboxylate lies near the 5' phosphate (4–5 Å) with a bridging water molecule in between, analogous to Asp 424 in Pol I (Fig. 3). The most obvious differences between the two enzymes include the two arginine side chains binding to the 5' phosphate in staphylococcal nuclease in contrast with one tyrosine hydroxyl in Pol I. Nevertheless, some aspects of the chemical mechanisms of DNA hydrolysis may be similar in these two enzymes even though their overall structures are completely unrelated.

Attempts to bind deoxynucleoside triphosphates to the enzyme in these crystals has failed thus far, presumably because of the high ionic strength of the 70% saturated ammonium sulphate required to stabilize the crystals.

DNA binding site

The size and shape of the large cleft suggests that it binds the duplex product of DNA synthesis. B-DNA fits into the cleft if the J and K α -helices are placed partially into a major groove (Fig. 4). Once again, although there is a structural and functional analogy between the J and K helices and the two-helix motif common to some regulatory proteins^{20–23}, there does not seem to be a strong structural homology. The two helices may function in Pol I to fix the translational position of the DNA on the protein, as sliding of the polymerase would require the protein to spiral along the DNA. One side of the cleft is ~50 Å long

Table 1 Heavy-atom derivative preparation and refinement

Concentration (mM)			Resolution limits (Å)								
			11.7	8.3	6.4	5.2	4.4	3.8	3.4	3.0	Total
5-HgdUMP	4	fr.m.s./Er.m.s.	3.74	3.57	3.89	3.39	2.19	1.67	1.41	1.18	1.95
		R _c	0.29	0.38	0.39	0.42	0.69	0.74	0.67	0.72	0.57
K ₂ Au(CN) ₂	15	fr.m.s./Er.m.s.	1.77	1.73	1.89	1.66	1.15	1.02	—	—	1.36
		R _c	0.54	0.67	0.63	0.66	0.86	0.85	—	—	0.74
K ₂ Pt(CN) ₄	50	fr.m.s./Er.m.s.	1.82	1.42	1.71	1.61	1.30	1.13	1.04	—	1.32
		R _c	0.59	0.69	0.71	0.86	0.77	0.84	0.88	—	0.79
K ₂ Pt(NO ₂) ₄	18	fr.m.s./Er.m.s.	0.91	1.22	1.62	1.55	1.17	1.21	1.26	1.23	1.26
		R _c	0.80	0.75	0.86	0.75	0.90	0.82	0.86	0.91	0.83
		Number of reflections:	255	576	1,002	1,593	2,279	3,087	4,014	4,971	17,777
		Mean figure of merit:	0.91	0.95	0.92	0.86	0.78	0.73	0.59	0.46	0.66

Abbreviations: fr.m.s., the root mean square calculated heavy-atom structure factor; Er.m.s., the root mean square lack of closure, determined from the centric reflections only; R_c , the centric R -factor given by $R_c = \frac{\|F_{ph} - F_p\| - |f_h|}{\|F_{ph} - F_p\|}$, where F_{ph} and F_p are the observed structure factor amplitudes for the derivative and parent crystals respectively and f_h is the calculated heavy-atom structure factor amplitude. All soaking conditions included 70% saturated ammonium sulphate, 1 mM ZnSO_4 , 20 mM MgSO_4 , 14 mM dTMP and 66 mM PIPES, pH 7.0, except for the 5-Hg-dUMP, which did not contain dTMP.

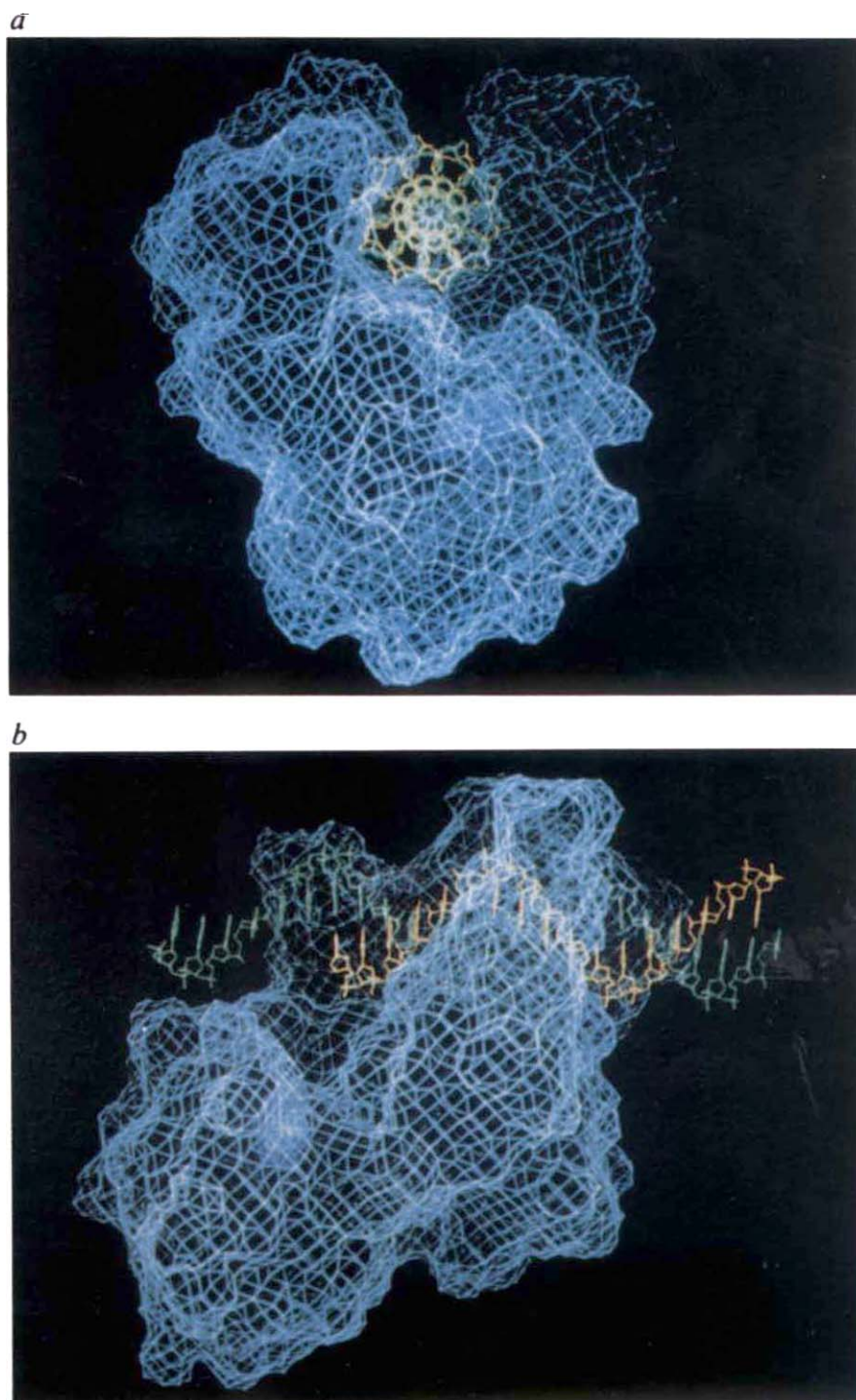


Fig. 5 A space-filling representation of large fragment complexed with DNA, viewed (a) down and (b) perpendicular to DNA helix axis. The solvent-accessible surface of the protein is represented by the net and was generated using an algorithm of Lee and Richards³⁰ and displayed as programmed by M. Hanschumacher. The green strand of the DNA is the template strand and the yellow strand the primer strand. The dot surface represents the van der Waals' radii of dTMP.

and can serve to orient the DNA helix axis. It can also bind the single-stranded template. Although the cleft as it exists in these crystals is a few Ångströms too narrow in some places, steric overlap of protein and DNA can be alleviated by adjusting the positions of protein side chains and slight movement of the helical protrusions (Fig. 1; I, H, J and K).

The structure of Pol I suggests a mechanism for its processivity, that is, its ability to incorporate many nucleotides into DNA before it dissociates from the DNA substrate²⁴, as is common in other polymerase reactions²⁵. The location of a flexibly attached 50-residue subdomain at the tip of the I and H helices in our model suggests that this small subdomain closes off the cleft after DNA binds. Thus, if this protein does enclose its DNA substrate, the rate of dissociation of the DNA product may be very slow and, if significantly slower than the rate of polymerization, could result in processive polymerization. The

enzyme may simply slide to the new 3' terminus between polymerization steps.

Several observations support this suggested location of DNA on the protein. First, the position of the amino acid change in *polA6*, a mutant Pol I defective in DNA binding²⁶, is consistent with our DNA complex. This point mutation is a change of Arg 690 to His (C. Joyce, personal communication) and we show here that this Arg is close to the DNA (Fig. 6). As it is making a salt link with the C-terminal α -carboxylate, Arg 690 is important in maintaining the structural integrity of the two-helix protrusion involved in binding DNA in our model. The *polA5* mutation changes Gly 850 to Arg (C. Joyce, personal communication) with the result that the enzyme is less processive on the DNA substrate²⁷; also, Gly 850 is near the proposed DNA binding site. Second, the high degree of amino acid sequence conservation between Pol I and T7 DNA polymerase



Fig. 6 Stereo drawing of positions of the *polA6* and *polA5* mutations. On the α -carbon backbone of the large, C-terminal domain of the Klenow fragment and the phosphorus atoms of the model of B-DNA are superimposed Arg 690 (which becomes His in *polA6*) and Gly 850 (which becomes Arg in *polA5*) (C. Joyce, personal communication).

in those polypeptides forming the surface of the cleft²⁸ suggests an important functional role for the cleft. Finally, calculation of the electrostatic charge potential using these incomplete coordinates shows a positive potential only in the cleft and a negative potential elsewhere (J. Warwicker, D.L.O. and T.A.S., unpublished data).

Although we have no evidence about the location of the 3' end of the primer strand, the suggested position in Fig. 4 seems plausible. The primer terminus was positioned to: (1) be as close as possible to dTMP; (2) interact with the protein; (3) allow interactions between the resulting single-stranded template strand and the protein and (4) to be on the end of the DNA that is nearest the N-terminus, as the small fragment removed from the N-terminus is required for nick translation. As an alternative, we tried to build an enzyme-DNA model with the 3' terminus of DNA attached to the observed position of the nucleoside monophosphate. No model which allowed extensive protein-DNA interactions could be constructed, nor was it possible to fit the DNA into the cleft. In the orientation shown in Fig. 4, several bases of the single-stranded template strand can make interactions with the protein.

The observed binding site of dTMP and the proposed location of the primer terminus are too far apart for the enzyme to be functional in exonuclease activity in this conformation and with these positions of the substrates. The 5' phosphate of dTMP is about 25 Å from the primer terminus shown in Fig. 4. There are several explanations of this large distance; first, the binding of a suitable DNA substrate to this enzyme may result in a large change in the conformation of the enzyme. The dTMP could be brought into contact with the DNA by a change in the relative orientation of the two domains, a conformational change analogous to the change induced by the binding of glucose to hexokinase²⁹, although somewhat larger. Second, the binding of DNA may differ in the polymerase and exonuclease reactions.

The DNA may not bind in the cleft for the exonuclease reaction, but may bind instead only to the small domain. Third, if three or four bases become unpaired at the 3' terminus in addition to the mismatched base, the short single-stranded section formed would span the distance between the dNMP site and the double-stranded DNA in the cleft.

To assess the changes in protein conformation resulting from binding of the DNA substrate and to locate the position of the dNTP substrate in a ternary complex, we next seek to crystallize the protein with a suitable DNA.

Conclusions

The crystal structure of the large proteolytic fragment of DNA polymerase I has a striking and unique shape suggesting some aspects of its function. Deoxynucleoside monophosphate binds to the smaller of two domains, with its 5' phosphate interacting with a tightly bound metal ion; detailed analysis of this enzyme-product complex may elucidate the enzymatic mechanism of the exonuclease reaction and perhaps the process of 'editing' by which this exonuclease activity increases the fidelity of DNA synthesis. The hand-like shape of the large domain may form a suitable binding site for the DNA substrate of the polymerase reaction. A flexible subdomain may allow the enzyme to surround the DNA substrate completely and thereby play an important part in processive nucleotide addition. The locations of the primer terminus and the nucleoside triphosphate in a productive ternary complex remain to be elucidated.

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Ultra-high energy γ rays and cosmic rays from accreting degenerate stars

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Ultra-high energy (UHE) γ -ray emission, with photon energies up to 2×10^{16} eV, has been detected^{1,2} from the galactic X-ray source Cygnus X-3 (Cyg X-3), implying the acceleration of charged particles there to even greater energies. We present here a model for acceleration of particles to such energies in binary-star systems containing accreting magnetized neutron stars, which, if correct, implies that many of the known pulsating X-ray sources and magnetized cataclysmic variables should also emit UHE γ rays.

Cyg X-3 is a remarkable X-ray source³, emitting X rays of energy ≥ 1 keV, with a luminosity $\sim 3 \times 10^{38}$ erg s⁻¹, which are modulated with a 4.8-h period, thought to be the orbital period of the underlying binary-star system (though there is no direct spectroscopic evidence to support this conclusion). The SAS II satellite detected⁴ the source in the 30–100-MeV range, also showing the 4.8-h period, but the COS-B satellite did not do so two years later in a similar energy range⁵.

For energies $\geq 10^{15}$ eV, there have been two independent reports of γ rays from Cyg X-3, again modulated with the 4.8-h period. First, Samorski and Stamm¹, using the Kiel extensive air shower array, during 1976–80, detected a flux of photons with energies between 2×10^{15} and 2×10^{16} eV. For a source distance of 10 kpc the flux is $\sim 5 \times 10^{36}$ erg s⁻¹. Second, Lloyd-Evans *et al.*² reported γ rays at energies of 2×10^{15} eV. The flux from soft γ -ray energies up to the highest observed energies shows a continuous spectrum, with a power law of spectral index -1.1 , steepening sharply at energies $\geq 10^{16}$ eV.

We are aware of only one attempt to explain the production of UHE γ rays from Cyg X-3. Eichler and Vestrand⁶ proposed that the origin of the radiation is associated with a rapidly rotating strongly magnetic neutron star in a binary-star system with orbital period of 4.8 h. For a neutron star with a magnetic field $\sim 10^{12}$ G and rotating with a period of ~ 10 ms, it is possible to achieve potential gaps near the pulsar to accelerate ions to energies $\sim 10^{16}$ eV. Although the energy source in this model is the rotational energy of the neutron star, the total luminosity of the source is comparable to the Eddington luminosity, suggesting that accretion is the energy source. Here we base an alternative model, in which the energy source is accretion, on a unipolar inductor model proposed to accelerate particles in active galactic nuclei by accretion onto massive black holes^{7–11}.

Consider the accretion of ionized matter onto a degenerate star of mass M and radius R . If the accreting matter forms a differentially rotating accretion disk around the degenerate star, the ambient magnetic field becomes amplified and a poloidal magnetic field of the form $(B_r, 0, B_z)$, in cylindrical polar co-ordinates, is then formed⁷, where $B_z(r) \propto r^{-1/2}$. If the accreting material is highly conducting, an observer in a non-rotating inertial frame will see an electric field $E_r(r) = -u_\phi(r)B_z(r)/c$, where $u_\phi(r) = (GM/r)^{1/2}$ is the keplerian velocity of the disk material. If the inner radius of the disk is r_1 and its outer radius is r_2 , the potential difference across the disk is given by⁷

$$V = -\frac{(GM)^{1/2}}{c} B_z(r_1) r_1^{1/2} \ln(r_2/r_1). \quad (1)$$

(Note that in gaussian c.g.s. units, V is given in statvolts, where 1 statvolt = 300 V.) If the degenerate star is magnetized and has a dipole field whose axis is parallel to the rotation axis, then r_1

is given by the magnetospheric (or Alfvén) radius

$$r_1 = 1.8 \times 10^8 B_{12}^{4/7} R_6^{10/7} (M/M_\odot)^{1/7} L_{38}^{-2/7} \text{ cm} \quad (2)$$

where B is the surface magnetic field of the degenerate star and the total accretion luminosity $L = GMM/R$, where M is the accretion rate. Here $B_{12} = 10^{-12}$ G, $L_{38} = 10^{-38}$ L erg s⁻¹ and $R_6 = 10^{-6}$ R cm. A tacit assumption is that the magnetic field will remain anchored in the disk and create the required large-scale structure. If, however, buoyancy forces cause the flux tubes to rise to the low-density 'skin' of the disk, with resulting chaotic dynamo activity, the potential developed could be reduced. To determine whether this is possible requires detailed analysis.

If we further suppose that $B_z(r_1) \approx B(R/r_1)^3$, the value of the star's dipole field at $r = r_1$, then for a canonical neutron star with $B = 10^{12}$ G, $R = 10^6$ cm and $L = 10^{38}$ erg s⁻¹, it follows that $B_z(r_1) \sim 1.7 \times 10^5$ G and $V \sim 1.6 \times 10^{15}$ V if $r_2 \sim 10^{11}$ cm, which is interesting because the high potential difference occurs in regions of the accretion disk where the magnetic field is much lower than at the surface of the neutron star. Furthermore, from equations (1) and (2) it follows, for a dipole field, that $B_z(r_1) r_1^{1/2} \propto B^{-3/7}$. Hence lower surface magnetic fields produce higher values of $B_z(r_1)$, as r_1 is lower (but must be $> R$), and therefore higher potential differences across the disk. Thus the maximum voltage, $V_{\max}$, that can be generated occurs with $r_1 \sim R$ and the luminosity equal to the Eddington luminosity $L_E = 1.3 \times 10^{38} (M/M_\odot) \text{ erg s}^{-1}$. Hence, for $M = 1 M_\odot$, $L = L_E$, one finds $B = 1.3 \times 10^8$ G and $V_{\max} = 2 \times 10^{17}$ V, based on a simple application of Lovelace's model⁷. If $B_z(r_1)$ drops off faster than $r^{-1/2}$, the value of $V_{\max}$ would be reduced; thus for a r^{-1} dependence, the value for $V_{\max}$ would be reduced by a factor of ~ 2 .

The electrodynamic models^{7–11} suggest that jets of plasma can be created. The electrodynamic coupling between the generator (disk) and load (jet) can lead to storage and discharge of magnetic energy on time scales of $\sim t_s$ and t_d respectively¹¹. Hence the discharge potentials would exceed $V_{\max}$ by a factor $t_s/t_d \geq 1$. Although the value of t_s/t_d for Cyg X-3 is unknown, the X-ray variability of active galactic nuclei indicates¹¹ that for them $t_s/t_d \sim 100$.

The high voltages generated produce acceleration of charged particles in the $\pm z$ directions. If the angular velocity Ω of the disk is such that $\Omega \cdot \mathbf{B} > 0$, electrons are accelerated; if $\Omega \cdot \mathbf{B} < 0$, protons or ions are accelerated. The total current of charged particles is¹⁰ $I = \epsilon c V$, where $\epsilon \ll 1$, and hence the energy output in the two particle beams is

$$L_{\text{part}} \approx 2\epsilon c V^2 \approx 2\epsilon \frac{GM}{c} B_z^2(r_1) r_1 (\ln(r_2/r_1))^2 \quad (3)$$

which must satisfy the condition $L_{\text{part}} \leq (GM/r_1) \dot{M} = LR/r_1$.

We now apply these results to the Cyg X-3 system. Assuming that the 4.8-h period represents the binary orbital period, the disk can extend to $r_2 \sim 10^{11}$ cm. Setting the accretion luminosity equal to the observed X-ray luminosity, $L_{38} \approx 3$, implies a neutron star of mass $M \approx 2 M_\odot$ accreting at its Eddington limit. As above, taking $r_1 = R = 10^6$ cm, we find $B = 1.6 \times 10^8$ G and $V_{\max} = 3 \times 10^{17}$ V. Noting that $L_y/L_x \approx 2 \times 10^{-2}$, where L_y is the UHE γ -ray flux, and $L_E \geq L_{\text{part}} \geq L_y$, one finds $5 \times 10^{-3} \geq \epsilon \geq 10^{-4}$. It is interesting that a neutron star with a magnetic field $\sim 10^8$ G is probably spinning rapidly with a period of ~ 1 ms; it could either have been born with a weak magnetic field and spinning rapidly, or have been spun up by accretion after an initially stronger magnetic field had decayed. Thus, Cyg X-3 corresponds to a progenitor phase in some evolutionary scenarios for the formation of a millisecond pulsar¹². The rapid rotation and weak magnetic field implies that the binary would not resemble a standard binary X-ray pulsar as is the case for Cyg X-3.

How do these charged particles manifest themselves as the observed γ rays with energies $> 10^{16}$ eV? If the process results

in acceleration of positively charged particles (mostly protons), then protons of energy $\sim 2 \times 10^{17}$ eV are accelerated in Cyg X-3. Given such particles, they can then form^{6,13} π^0 -decay γ rays by proton-proton collision with gas in the atmosphere of the companion star, or with other circumstellar gaseous material. Only a few per cent of the total fast-particle luminosity need then be converted into photons, a factor easily accommodated by the pion production mechanism. The resulting π^0 -decay γ rays will have energies of the order 10^{16} eV. Other radiative processes, of course, will come into play, but will not be considered here.

In the above discussion, we have assumed for simplicity that the particle beams are directed perpendicular to the disk. Such symmetrical emission is unlikely to result in significant variation in the γ -ray flux with a period of 4.8 h, but a highly-inclined neutron star magnetic axis is likely to produce a sufficient degree of asymmetry in the particle beams for eclipse-like intensity modulations to occur, as are indeed observed.

This model has several other consequences which we list here without detailed comment:

(1) Depending on the sign of $\Omega \cdot B$, either electrons or protons will be accelerated. Most systems, of course, should have an arbitrary angle between Ω and B . Nonetheless, many accreting systems containing magnetized collapsed objects should also be UHE γ -ray sources and indeed, two other accreting binary X-ray sources containing neutron stars, Her X-1 and Vela X-1, have been reported as such. Thus, Her X-1 has been seen¹⁴ at energies of 10^{12} eV and, assuming its magnetic field is of the order 10^{12} G, the present model predicts that it would radiate at photon energies up to a maximum of $\sim 10^{15}$ eV. The γ rays are pulsed with the rotational period ($= 1.24$ s) of the neutron star, which suggests, in our model, that the high-energy ions produced in the disk are modulated, with this period, by interaction with the X rays beamed by the accreting neutron star. The peak γ -ray luminosity is comparable to the X-ray luminosity of $\sim 10^{37}$ erg s⁻¹. Such a high luminosity cannot be explained by rotational energy loss⁶ from such a slowly rotating pulsar and therefore strongly supports our model where the γ -ray luminosity is derived from accretion. Vela X-1, observed¹⁵ at energies up to 2×10^{15} eV and pulsed with the orbital period of 9 days, has an unknown magnetic field strength, but would fit into the present model with a surface magnetic field of the order 10^9 G. As the neutron star in Vela X-1 has such a slow rotation period of 283 s, the observed γ rays cannot be explained by the model of ref. 6. Observational support for these two objects as UHE sources, however, awaits independent confirmation.

(2) A number of cataclysmic variables containing magnetized white dwarfs¹⁶ should also act as unipolar inductors^{7-11,17}, accelerating fast particles. For example, V1223 Sgr, which contains a white dwarf with a magnetic field $B \sim 10^6$ G and a luminosity $L \sim 5 \times 10^{34}$ erg s⁻¹, should produce $\sim 3 \times 10^{13}$ V if $M = 1 M_\odot$ and $R = 5 \times 10^8$ cm.

(3) This process has two very appealing features which make it a strong candidate for the source of UHE galactic cosmic rays. First, large electric potentials can develop over extended regions of relatively weak magnetic fields. Thus, unlike acceleration near a neutron star surface, accelerated particles can escape the accelerating region without significant loss of energy. Second, as accretion is the ultimate energy source, the source can last for time scales that are astronomically significant.

In conclusion, the UHE γ rays reported to have been observed from Cyg X-3 (refs 1, 2), Her X-1 (ref. 14) and Vela X-1 (ref. 15) cannot all be explained in a model where the luminosity is derived from the rotational energy⁶ of a neutron star. We suggest instead that the high-energy particles, which produce the rays, are accelerated in an accretion disk, surrounding a neutron star, by the unipolar inductor mechanism⁷⁻¹¹; thus accretion is the ultimate energy source.

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Transient quasi-periodic oscillations in the X-ray flux of Cygnus X-3

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Cygnus X-3, a bright X-ray source at a distance¹ of more than ~ 12 kpc beyond the edge of the galactic plane, has peculiar properties including very strong radio outbursts², possibly showing jet-like features³, and emission in various γ -ray bands⁴⁻⁶ (but see ref. 7). The discovery of its 4.8-h X-ray period⁸ showed that the source is probably a compact ($\sim 10^{11}$ cm) binary system, but its nature and that of its companion, the mode of energy generation (accretion or Crab-like pulsar) and also the cause of the X-ray modulation are still uncertain. We describe here X-ray data on Cyg X-3, obtained with EXOSAT, showing transient quasi-periodic oscillations with amplitudes between 5 and 20% of the 1-10-keV flux and periods in the range 50-1,500 s. The oscillations persisted typically for 5-40 (quasi-oscillation) cycles, occurred exclusively in the phase interval 0.0-0.75 (rising branch and top of the X-ray light curve), were seen during the high state and probably also during the low state of the source, and, at least once, were observed with two different periods simultaneously. We suggest that the quasi-periodic flux variations are caused by quasi-periodic phenomena in an accretion disk which partially occults the X-ray source. This tentative interpretation is in accordance with the idea⁹ that the 4.8-h modulation of the X-ray flux in Cyg X-3 arises by partial occultations of the X-ray source by an accretion disk, like in low-mass X-ray binaries such as 4U1822-37.

The X-ray intensity of Cyg X-3 shows a smooth asymmetric modulation¹⁰, remarkably constant in shape on long time scales¹¹, but fluctuating strongly from cycle to cycle¹², mainly on time scales $< 3,000$ s (ref. 12). To study the short-term variability of Cyg X-3, we have observed the source with EXOSAT on six occasions between October 1983 and May 1984. We report the intensity variations observed with the Medium Energy (ME) experiment¹³ on time scales > 5 s. Observations with durations between 0.7 and 2.5×10^4 s were performed on 22 October, 5 November, 3 and 18 December 1983 and on 3 January and 21 May 1984. During two of the observations the source was in a low state⁹ with a background-subtracted counting rate (during light-curve maximum) between 180 and 260 count s⁻¹ (1-10 keV) in the full 1,500-cm² ME-array; in the remaining four observations the light-curve maximum count rate varied between 830 and 670 count s⁻¹. On intensity plots of the data, transient quasi-periodic oscillations were immediately apparent in most,

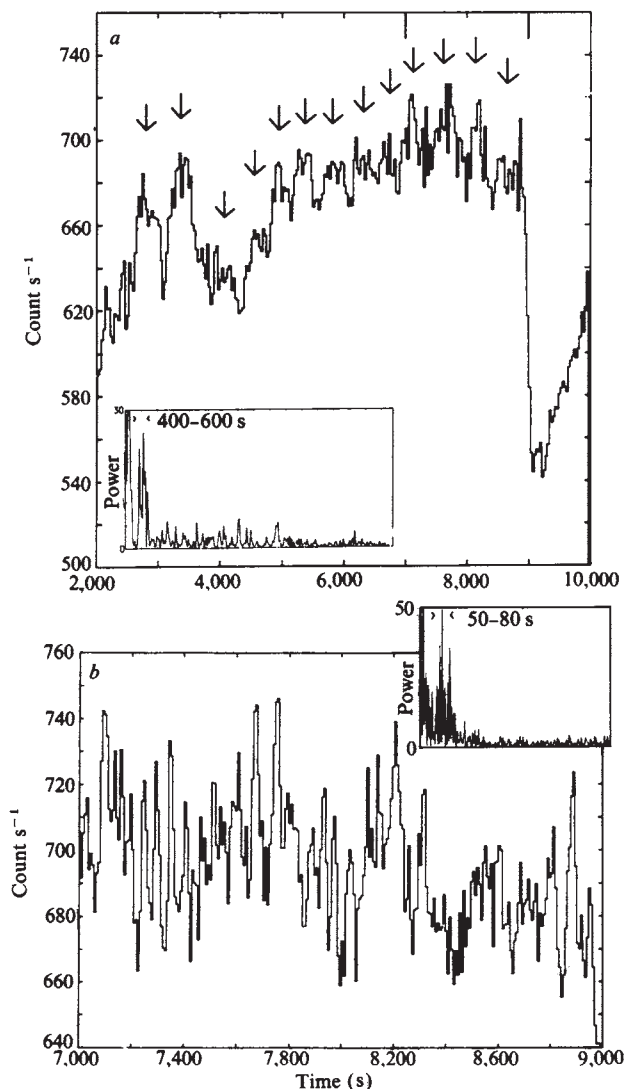


Fig. 1 Composite quasi-periodic oscillations observed in the 1–10-keV flux of Cyg X-3 on 5 November 1983. *a*, Counting rate in the 1,500-cm² ME array in 30-s bins. The statistical accuracy per bin is 5 count s⁻¹. The peaks of the ~500-s oscillations are indicated with arrows. Notice the sharp drop in flux near the end of the plot. *b*, An expansion of the data immediately preceding the drop in flux, at 4× greater time resolution, revealing a series of quasi-periodic flares with recurrence times between 50 and 80 s. Insets show the power spectra of the data, detrended by subtracting a polynomial to remove low-frequency components. The power was normalized to a mean level of one in the high-frequency range. Counts are uncorrected for the ~30 count s⁻¹ background level.

though not all, of our observations. They therefore may be a common aspect of the flux variations in Cyg X-3.

Figures 1 and 2 show the 1–10-keV flux of the source in the high state during the intervals when the oscillations were observed. On 5 November 1983, the flux behaviour is particularly interesting. About 2,000 s after the start of the observation (that is, at binary phase 0.1, with phase 0.0 at light-curve minimum), quasi-periodic oscillations with a typical period of 500 s begin and persist until the sharp 20% drop in source flux at $t = 9,000$ s which takes place within ~100 s (Fig. 1*a*). During the 2,000 s preceding the drop, a second quasi-periodic oscillation with a ~70-s period is present (Fig. 1*b*), so that during this time two oscillations occur simultaneously. (Note that the time scales of the sharp intensity drop and the preceding oscillation are quite similar.) On 3 January 1984, we observe initially a ~250-s oscillation just after light-curve minimum, which modulates the source flux by up to 20% (Fig. 2*c*); thereafter the source settles in a slower 1,400-s 7% quasi-periodic oscillation during the rise to light-curve maximum (Fig. 2*d*).

Power spectra of the data (Figs 1 and 2 insets) show clearly the signature of the quasi-periodicities and the typical periods of the oscillations. Note the wide range in coherency: from 'coherent' within the observational accuracy, on 22 October 1983 (all the power in one bin in the power spectrum, that is, $\Delta f/f < 4\%$) to a considerable frequency spread of power on 5 November 1983.

There is no generally accepted method for estimating the significance level of quasi-oscillations observed in a variable source. For coherent oscillations a common approach is to compare the power in the oscillation with the mean power in a local frequency range (which contains the oscillation) and to assume an exponential distribution of the local power amplitudes (see, for example, ref. 14). If we compare in our data the highest power in the enhancements caused by the quasi-oscillations with the mean power in the regions next to the enhancements, we find significance levels of 4–6 σ in all cases except on 18 December 1983 (2 σ). This method is rather insensitive to quasi-oscillations in general, however, as it neglects a large fraction of the power (usually distributed over several bins in the power spectrum), and is therefore expected to underestimate the significance of the quasi-oscillations. (Note that only a small number of bins in the power spectrum is 'searched' in this procedure, as the approximate period of the quasi-oscillation is known previously from the intensity plots.) The power spectra show a marked increase towards lower frequencies ('red noise') on which the peaks caused by the quasi-oscillations are superimposed and hence an estimate of the local mean power from the adjacent regions may be inaccurate. The low-frequency noise in Cyg X-3 has been measured accurately during a long (30 h) uninterrupted EXOSAT observation (R. Willingale, A. R. King and K. A. Pounds, personal communication) of the source in February 1983. Using this to estimate the local mean power underlying the quasi-oscillations (assuming the amplitude of the low-frequency noise to be proportional to the source flux), we find in all but one case (400–600 s, 5 November 1983) a somewhat higher significance.

The source was in a low state during our 21 May 1984 observation. In the rise to light-curve maximum, we observe a series of flares and dips (Fig. 3), which, according to the Fourier analysis of the data, has a periodic component of ~1,000 s. Because of the similarity with the high-state observations, we conclude that the quasi-periodic oscillations probably occur also during the low state, although we do not consider this result as yet established.

In most models^{9,15–18} for Cyg X-3, the X-ray source is surrounded by a scattering region. The ~100-s 20% drop in source flux, observed on 5 November 1983, allows us to place an upper limit of $\sim 1.5 \times 10^{12}$ cm on the radius of this region if its X-ray optical depth is > 1.6 . This limit is mainly relevant to cocoon¹⁵ and stellar wind^{16,18} models of the source.

The observed time scales of the quasi-periodic oscillations (50–1,500 s) are orders of magnitude shorter than the relevant time scales for changes in structures of the size of the binary system (or larger) and correspond to the dynamical time scale of matter well inside the Roche lobe of the X-ray source. In a standard disk model, the rotation time of the matter in the disk, the time scale on which hydrostatic equilibrium in the z -direction is approached and, for a value of the viscosity parameter α of order 1, the thermal time scale are (at the same radius r in the disk) all of the same order¹⁹. For a 1.4 $M_{\odot}$ neutron star, the observed oscillation time scales correspond to values of r between 5 and 50% of the radius of a Roche-lobe filling disk. Therefore, we tentatively suggest the oscillations are caused by partial occultations of the X-ray source by matter at different radii in an accretion disk. The transient quasi-periodicities could arise by oscillations in the z -direction, or by structures projecting above the surface of the disk ('blobs'), which are carried around by the rotation of the matter in the disk. In several cases (for example, 5 November, 2 January) the dips of the observed oscillations are of considerably shorter duration than the peaks, which would seem to favour the latter mechanism (a disk oscilla-

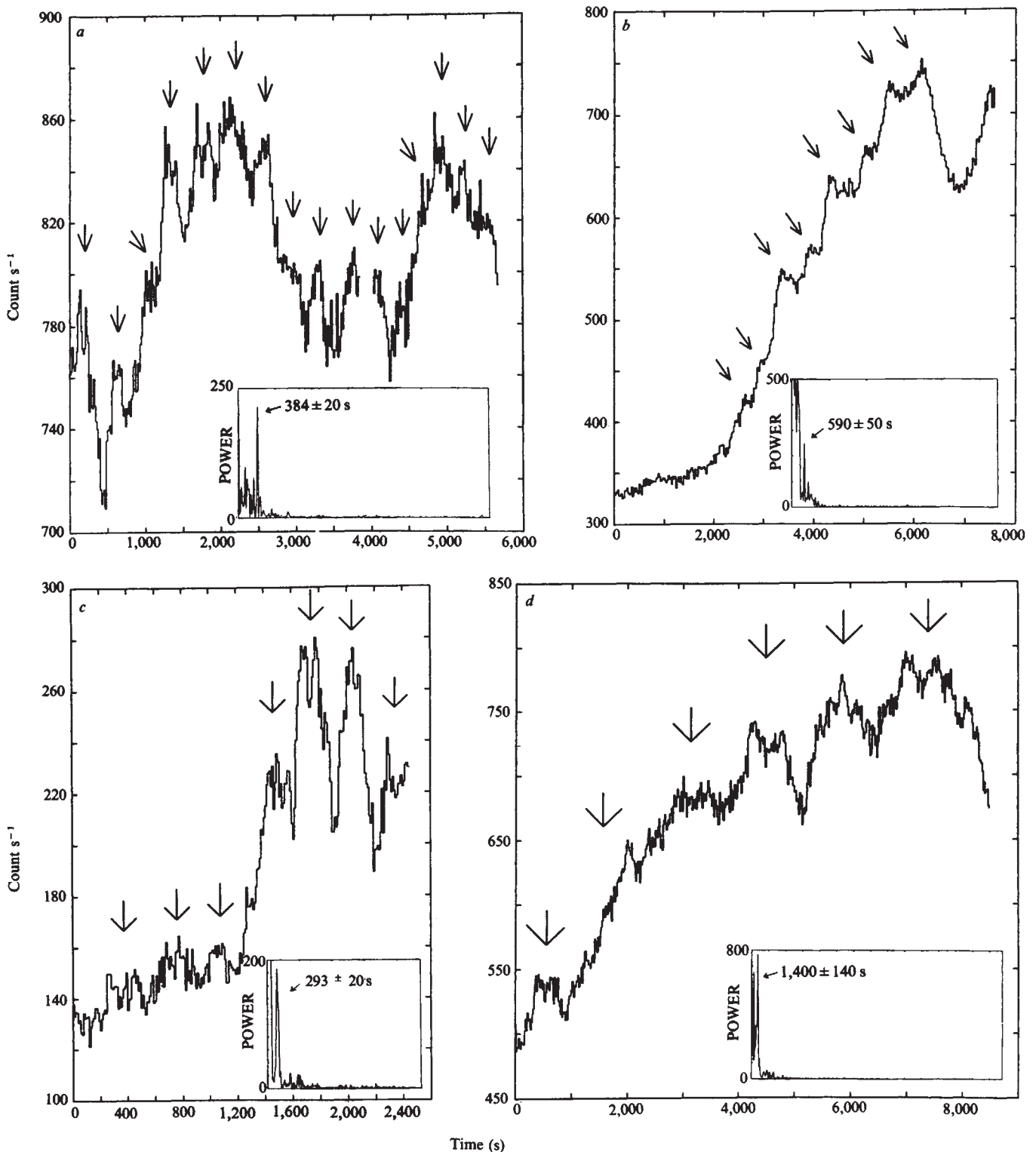


Fig. 2 Quasi-periodic oscillations observed during three different high-state observations. Bin sizes for *a-d* are 15 s (22 October 1983), 22.5 s (18 December 1983), 10 and 15 s (3 January 1984), respectively. Collecting area was 1,500 cm² and background level (not subtracted) ~30 counts s⁻¹, except for *c*, where these values are a factor of two less. Power spectra (insets) are as in Fig. 1. Note the different scales of *a-d*.

tion would be expected naively to give rise to a sinusoidal modulation) with the blobs being of relatively small azimuthal extent ($< 90^\circ$). Depending on the radial extent and the nature of the blobs (ripples on the disk surface²⁰, magnetic loops), shearing would destroy them after some time¹⁹, consistent with the transient nature of the quasi-oscillations.

This interpretation is in accordance with the accretion-disk corona model which has been applied⁹ to Cyg X-3 as well as

to the low-mass X-ray binaries 4U1822-37 and 4U2129+47. Similar explanations have been proposed for a 10³-s quasi-periodicity observed in the pulsating source 4U1626-67²¹ and recently for fluctuations seen²² in 4U1755-33²³ and 4U1705-44²⁴. These sources are all low-mass X-ray binaries thought to be powered by the Roche-lobe overflow of a red-dwarf companion to the X-ray source. The observed gradual lengthening of the orbital period²⁵ of Cyg X-3 probably implies orbital

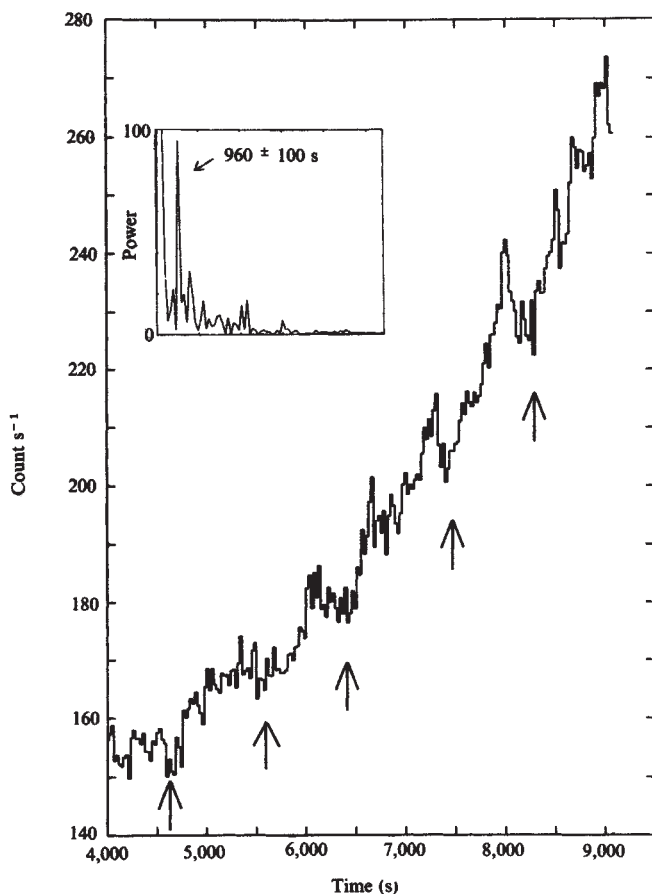


Fig. 3 As Fig. 2, but for the low state observed on 21 May 1984. Bin size is 24 s. The dips which contribute to the periodicity showing up in the power spectrum (inset) are indicated.

expansion, which would prevent a hypothetical red dwarf from overflowing its Roche lobe were it to follow approximately the mass-radius relation for low-mass main-sequence stars during its mass loss. Moreover, Cyg X-3 has very peculiar radio and possibly γ -ray emission properties. It is thus quite different from any of the low-mass X-ray binaries mentioned above, but the way in which their X-ray light curves are produced may well be similar.

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Transport of venusian rolling 'stones' by wind?

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Speculation that aeolian processes operate on Venus^{1,2} has been confirmed tentatively by analyses of results from Venera spacecraft³⁻⁵. Here we describe simulations of venusian wind processes, which show that particles are moved by 'rolling' at wind speeds as much as 30% lower than those required for saltation threshold. This mode of wind transport is only observed for sustained periods in water on Earth; thus, there are similarities between aqueous fluid transport on Earth and atmospheric transport on Venus. The formation of small sand ridges and grooves oriented parallel to the wind direction is associated with the rolling of grains in venusian simulations and these structures may be unique aeolian features on Venus.

Because the venusian atmosphere is almost 50 times denser than that of the Earth, wind speeds for particle movement are reduced by about an order of magnitude on Venus compared with Earth. Consequently, the measured near-surface winds of $0.5\text{--}1.0\text{ m s}^{-1}$ seem to be well within the range required to transport sand-size particles⁶ and aeolian processes may play a significant role in the modification of the surface, both in the formation of landforms, such as dunes, and in the redistribution of sediments. To determine the characteristics of aeolian processes on Venus, a wind tunnel (VWT) capable of simulating the near-surface venusian environment was fabricated. Previous experiments^{7,8} have established (1) saltation threshold wind speeds as a function of particle size, (2) the flux of saltating particles, (3) the speed of individual grains, and (4) the nature of small bedforms, such as ripples.

Bagnold⁹ has described three modes of aeolian transport on Earth: surface creep, saltation and suspension. Generally, surface creep involves coarse grains ($\leq 4,000\text{ }\mu\text{m}$), saltation involves coarse silt and sand grains ($\sim 40\text{--}4,000\text{ }\mu\text{m}$) and suspension involves fine particles ($< 40\text{ }\mu\text{m}$). As the size of grain most easily moved (that is, by weakest winds) is fine-medium sand ($\sim 100\text{ }\mu\text{m}$) most near-surface windblown particles are moved by saltation. Indeed, on Earth, both surface creep (called 'impact creep' by Sharp¹⁰) and suspension result primarily from the impact of saltating grains.

Bagnold⁹ noted that as wind speed increases over a bed of loose sand grains at rest, the direct pressure of the wind begins to roll some of the grains. At a distance of 30-40 cm downwind from the point where rolling begins, grains increase in speed and begin to saltate, frequently causing a cascading effect in which other grains are set directly into saltation on impact. In our experiments, conducted under terrestrial conditions¹¹, we find a similar sequence and note that the transition from rolling to fully developed saltation is rather abrupt and the rolling stage is very brief (less than a few seconds), despite careful control of the wind speed. Thus, rolling as a mode of aeolian transport does not appear to be important on Earth in terms of mass transport of material, as it occurs either briefly on attainment of the threshold velocity or intermittently by saltation impact.

In venusian simulations, we ran six sizes of well-sorted particles ranging from fine sand to small pebbles (average diameters are 105, 300, 800, 4,600, 7,800 and $13,000\text{ }\mu\text{m}$; the three smaller sizes were quartz, the three larger sizes were rounded grains of microcrystalline calcite). For each size, a bed of particles $\sim 1\text{ cm}$ thick was spread evenly over the test plate of the wind tunnel. The tunnel was pressurized to venusian conditions, run briefly at a wind speed above threshold to develop a natural aeolian-textured surface, then stopped⁷. The wind speed was then slowly increased while the test bed was observed through viewing ports and the motion and general behaviour of individual grains were

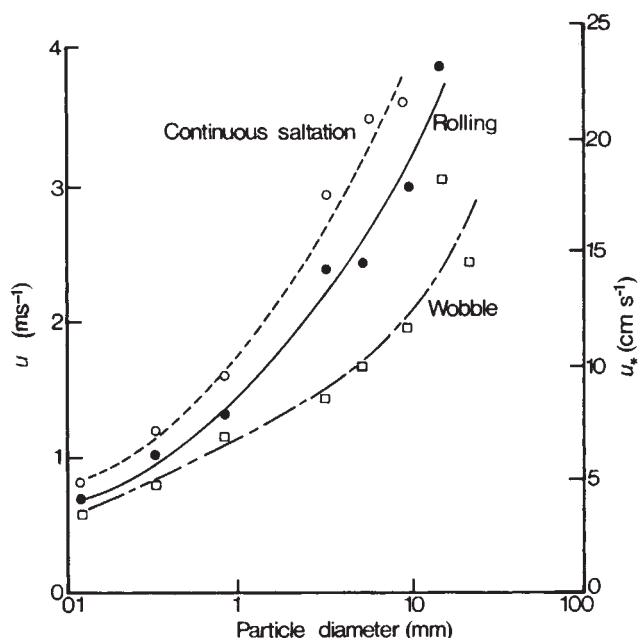


Fig. 1 Wind threshold curves for three types of particle motion in simulations of Venus: u_* is wind friction speed; u_∞ is 'freestream' wind tunnel speed, which can be taken as approximately equivalent to wind speeds measured by the Venera landers.

noted. In some experiments, high-speed (up to 4,500 frames per second) motion pictures were taken through side viewing ports to observe the motion of grains near the bed.

We observed three types of particle motion in these simulations of Venus: wobbling, rolling and fully developed saltation. Previous experiments⁷ showed that impact creep also occurs and, although experiments have not been conducted with fine particles, we presume that suspension would also occur on Venus. The threshold wind speeds at which the three types of motion are initiated are shown in Fig. 1 as functions of particle diameter. In wobbling motion, the grains quiver but did not move out of place. At a slightly higher wind speed, the grains began to tumble along the surface in the rolling mode and only a few grains would saltate. Continuous (fully developed) saltation did not occur until markedly high wind speeds were available, especially for large particles. The values shown on Fig. 1 for continuous saltation correspond to the single threshold curves predicted for Venus in previous studies^{1,2,6,7}. Thus, when the rolling mode of wind transport is taken into account, wind speeds on Venus for aeolian activity may be 30% less than previous estimates.

The rolling observed in VWT does not lead to a cascading effect, which on Earth ordinarily transforms the surface of the bed rather suddenly into a saltation cloud. Observations that the rolling mode can be maintained in VWT for an indefinite period and that a windspeed can be chosen (for any specified particle size) to maintain rolling are indications that venusian aeolian transport differs significantly from that on Earth. The sub-saltation regime of particle motion in VWT gives rise to a distinctive bedform parallel to the wind direction (Fig. 2). In the rolling mode, the test-bed surface was sculpted into a series of grooves and ridges oriented parallel to the wind for tests with the finest ($\sim 105\text{-}\mu\text{m}$) particles. These features, some of which extended the full length of the test plate, were several millimetres high by several millimetres wide and are remarkably similar in size and form to grooves and ridges produced in mud by unidirectional water currents¹². Allen suggests¹² that transverse components of flow associated with boundary-layer streaks are involved in eroding the grooves and shaping the bed. Above the saltation threshold, bedforms consist of small ripples and 'microdunes'¹⁸ that are transverse to the wind.

Our results show that the movement of particles in the dense

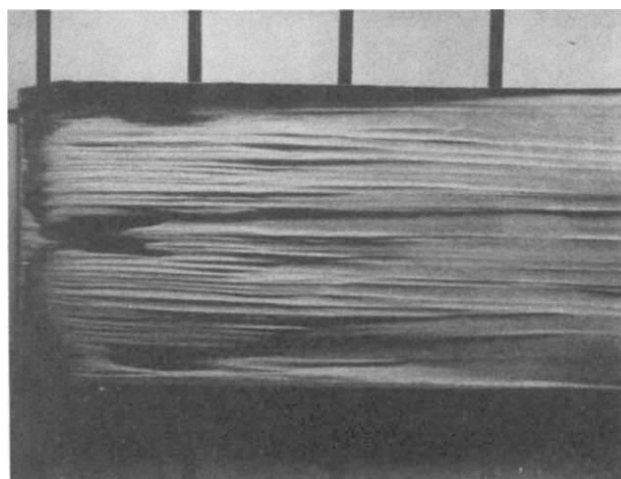


Fig. 2 View of grooves and ridges developed in fine quartz sand ($110\text{ }\mu\text{m}$ in diameter) in association with particles rolling along the surface in a simulation of Venus; the windspeed was held below the value required for continuous saltation. Area shown is about 20 by 40 cm; illumination from upper left; wind was blowing from left to right.

venusian atmosphere resembles that of water-driven particles on Earth. Bagnold⁹ compared particle movement in air with particle movement in water on Earth and noted that the fluid/particle density ratio is 1:2,000 in air and 1:2.65 in water; in the dense venusian atmosphere this ratio is approximately 1:40. Thus, aeolian conditions on Venus may share characteristics of both aeolian and aqueous conditions on Earth. For example, we have previously described small dunes (microdunes) that develop in venusian simulations and resemble sand waves formed in water but are absent in air on Earth⁸. In additional venusian simulations, we have observed the development of erosional slip faces on microdunes at high wind velocities (due to reverse flow in the lee eddy), similar to the erosional lee slopes developed in subaqueous bedforms referred to as current ripples¹³. Furthermore, Gilbert's description¹⁴ of the movement of particles in water is remarkably similar to our own observations from venusian simulations. He notes a rolling/saltation mode in which grains roll along the surface and occasionally saltate on short trajectories. Similarly, Abbott and Francis¹⁵ have recognized a distinctive rolling mode for small (centimetre) pebbles in water from high-speed photographs of flume experiments. They note, however, that rolling continues at fluid velocities well above the suspension threshold.

From comparisons with particle transport in water, we infer that the rolling mode is sustained in VWT because of the fluid-particle density ratio in the venusian environment. The fluid is evidently dense enough to generate surface shear for grain motion at velocities well below saltation threshold, similar to water, but, unlike water, is not sufficiently dense to carry the particles aloft in continuous saltation at low velocities.

The rolling mode of wind transport on Venus may be important from several considerations. (1) Frequency of transport: because threshold wind speeds for rolling are significantly lower than for continuous saltation, the occurrence of aeolian processes may be more frequent than is indicated solely on the basis of saltation threshold, despite the relatively gentle winds recorded on Venus. (2) Interpretation of venusian surface: if some bedforms are the consequence of a mode of fluid transport that is unique to the venusian environment, then interpretations of the surface should not rely solely on terrestrial aeolian (or water) analogues. (3) Movement of large particles: particles of 4–5 mm may be easily moved by rolling (Fig. 1), by winds within the range expected on Venus. (4) Flux of windblown material: previous estimates of the amount of material moved by the wind on Venus¹⁶ do not take into account the rolling mode, but are based solely on saltation. Thus, substantial material may be

moved by surface creep involving rolling (especially for large grains) at relatively low (sub-saturation) wind speeds. However, the total flux may still be far less than that of sediments transported by water on Earth¹⁷. (5) Small-scale surface features: small features transverse to the wind such as ripples and microdunes did not develop in experiments in which transport occurred solely by rolling. However, grooves and ridges parallel to the wind were formed and thus may be expected on Venus. The length of these features was governed by the size of the wind tunnel and it is possible that on Venus they could extend for tens or even hundreds of metres. Depending on the characteristics of radar imaging systems, such as wavelength, and the geometry of the radar beam, radar signatures from the surface of Venus could be influenced by the presence of these grooves and ridges. In these preliminary results, we note that although there is an association between rolling and longitudinal bedforms, the latter features are not necessarily a function of this mode of transport.

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Neutron generation in lightning bolts

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Intense electrical discharges through polymer fibres have been shown¹ to produce 2.45-MeV neutrons, probably by deuteron-deuteron fusion. Noting broad similarities between discharges in polymer fibres and natural lightning, Libby and Leukens² have suggested that neutrons are also generated in lightning flashes, as a result of the fusion of deuterium contained in the atmospheric water vapour; by rescaling the plasma parameters of polymer fibres to those involved in natural lightning, they have predicted a yield of $\sim 10^{15}$ neutrons per lightning flash. An experiment by Fleischer³, however, using fission track detectors placed near lightning arrestors, has failed to ascertain the neutron production in lightning discharges. Based on the number of background cosmic-ray tracks accumulated in these detectors during seven months of observations, Fleischer estimated an upper limit of 2.5×10^{10} neutrons per lightning stroke. In our experiment, we have attempted to keep the cosmic-ray neutron background at a negligible level by searching for neutrons from individual lightning strokes, for a time-interval comparable with the duration of the lightning stroke. Here we present the first experimental evidence that neutrons are generated in lightning discharges, with 10^7 - 10^{10} neutrons per stroke. Whether these neutrons are thermonuclear in origin, or are generated by non-thermal processes, remains to be determined.

Neutrons were detected at Gulmarg (altitude 2,743 m, latitude 34.07° N, longitude 74.42° E) by a low-energy cosmic-ray

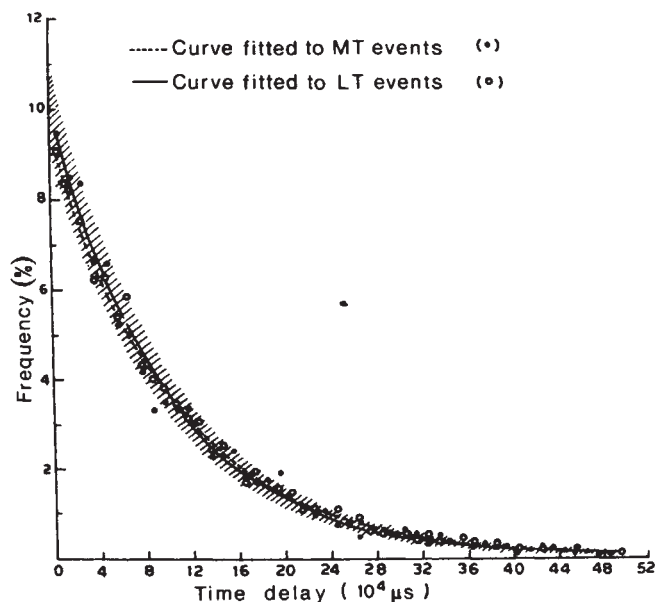


Fig. 1 The time-delay distributions of one-neutron lightning-triggered (LT) and manually-triggered (MT) events. Exponential curves fit both the data sets and produce a mean count rate of 10 neutrons s^{-1} , suggesting that these events are related to the background cosmic rays. The hatched portion represents the error range estimated for the fitted curves.

neutron monitor comprising 21 enriched BF_3 counters, each 90 cm long and 3.8 cm in diameter. The counters are arranged in the form of a pile and are placed over 28-cm-thick paraffin wax slabs 8 m above the ground, which helps render the monitor essentially opaque to low-energy background neutrons entering it from below. The counters are also covered from above with 7.5-cm-thick paraffin wax to thermalize 2.45-MeV neutrons and allow their detection with an efficiency of $\sim 3\%$. The monitor has an effective surface area of $3 \times 10^4 \text{ cm}^2$ and continuously records low-energy cosmic-ray background neutrons with an average rate of 1.8×10^4 neutrons per 30 min. The pressure coefficient for the monitor is -0.66% per mbar, in close agreement with reported values^{4,5}.

A linear antenna is mounted in the vicinity of the monitor to sense the short-term electric field variation associated with a lightning stroke. The signal, after suitable amplification and amplitude discrimination, activates an interval-counter to count 10- μs pulses from an oscillator, from the instant of the sensing of the electric field to the arrival of the first neutron detected in the pile. The time delay, thus registered in the arrival of the prompt neutron, is used for computing the distance to the lightning stroke, assuming a line-of-sight propagation. This neutron and those detected within the next 320 μs are recorded in four counters, each opened sequentially for an interval of 80 μs and having provision for counting a maximum of 99 neutrons. At the completion of the recording sequence, the time delay and the neutron counts are printed and the system primed for a subsequent lightning event after a dead-time of 400 ms. Note that the total counting time of 320 μs is large compared with the 50- μs duration of a typical lightning stroke and small compared with the average inter-stroke time of 40 ms⁶. The cosmic-ray background is negligible in this time and, thus, it is possible to monitor neutrons from individual lightning strokes with a high signal-to-noise ratio.

Between May 1980 and May 1983, the Gulmarg experiment has responded 11,200 times to potential gradient variations from lightning; it has also been activated manually on 8,400 occasions by feeding a pulse generator output to the lightning channel discriminator unit at randomly chosen epochs. Table 1 compares the frequency of the lightning-triggered (LT) events as a function of neutron number recorded per event with the corresponding

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number of manually triggered (MT) events. It is evident that, in a large majority of both LT and MT events, only one neutron is recorded within the monitoring interval of 320 μ s. Figure 1 shows, for these event groups, the distribution of the time-delays as recorded by the interval counter. Exponential curves fitting both data sets do not differ significantly from one another, suggesting a random arrival time of the detected neutrons in both cases. Further, the distributions result in an average time delay of 0.1 s between the arrival of successive neutrons, in agreement with the count rate of 10 neutrons s^{-1} recorded by the monitor for the background cosmic rays. These observations demonstrate clearly that the single neutron events result from the random arrival of cosmic-ray neutrons in the monitor and have no association with the preceding lightning discharge. The two-neutron events (see Table 1) are observed to constitute 2.23% and 1.11% of the total observed LT and MT events, respectively. These numbers are higher than the values calculated for the chance arrival of two neutrons from the cosmic-ray background, within a recording time of 320 μ s. It is conceivable that some of the two-neutron events result from the neutron bunches expected to be generated by the interaction of cosmic-ray hadrons of energy $\geq 10^{11}$ eV with the material present around the pile. At the same time, a possible association of these events with lightning flashes is suggested by the greater percentage of two-neutron LT events observed compared with the corresponding MT events.

Only for events of three or more neutrons are major differences observed between lightning and manual triggers. There are only four MT events with three neutrons and none with more than three neutrons. By contrast, 124 LT events have been registered with ≥ 3 neutrons, including 59 and 39 events with ≥ 6 and ≥ 9 neutrons, respectively, and one with a maximum count of 60 neutrons. The large differences in the relative number of LT and MT events and in the number of constituent neutrons show that most of the LT events with ≥ 3 neutrons cannot arise in the same manner as the four corresponding MT events. The processes precluded include neutrons from the cosmic-ray background, produced either singly, by low-energy primaries, or in bunches, by high-energy hadron interactions. The association of recorded neutrons with the cosmic-ray shower secondaries of primary energy $\geq 3 \times 10^{13}$ eV is also precluded, as only single neutron events were recorded when, in the course of a simultaneous run for 48 h, the system was triggered $\sim 2,800$ times by the output of a 10 m $\times$ 10 m shower array. The shower array boundary was ~ 4 m from the centre of the neutron pile and a finite number of shower triggerings should have been followed by a significant neutron count in the pile. That only single neutron events were recorded indicates the relative insensitivity of the neutron pile to high-energy shower neutrons. As the whole experiment is fully powered by batteries, any lightning-induced transients in the power lines are not expected to affect the operation of the pile. That the half-hourly counting rates of the monitor do not show any erratic behaviour further suggests that the lightning-generated electrical noise does not also adversely affect the operation of the system electronics, including the data recording unit. Moreover, it is not possible in this way to account for ≥ 3 -neutron events with time delays of 10 – 10^3 μ s, as the lightning-associated electrical noise should generally affect the recording system almost simultaneously with the activation of the lightning detector and thus be recorded as events with smaller time delays. That this is not the case, is clearly shown by our

data wherein, out of 124 LT events with ≥ 3 neutrons, 56 LT events have time-delays in the range 10^2 – 10^5 μ s.

The possibility of electromagnetic pick-up originating from subsequent strokes rather than from the first return stroke has been considered, although this is unlikely to happen, as the first stroke is usually the strongest stroke in a flash. One cannot, *a priori*, preclude the possibility of the system being triggered by strong electromagnetic activity preceding a lightning flash, whereas the neutron signal may be caused by pick-up from the lightning stroke. We have, therefore, verified experimentally how susceptible our system is to electromagnetic pick-up under natural lightning conditions. For this purpose, additional logic circuitry has been incorporated in the system, which is capable of detecting a maximum of four succeeding strokes of a lightning flash and recording their relative time spacings. This differential time information, together with the provision for recording the delay between the initial triggering of the system and the arrival of neutrons in four time-gates of 80 μ s each, would then reveal any direct connection between the neutron events and the lightning strokes. The experiment has been operated with this modification for about three months and several hundred LT events have been recorded, many of which were multi-stroke events. Whereas most of them were one-neutron events, a total of 24 LT events were two-neutron events and two LT events had three neutrons each. A comparison of the integrated and differential time information for the events reveals that in none were the detected neutrons in close coincidence with the detection of electromagnetic pulses from a single or a multi-stroke lightning flash, which reinforces our contention that the neutron signal cannot be attributed to any lightning-induced transients. It is therefore logical to associate the 124 LT events having ≥ 3 neutrons with neutron generation in lightning and to interpret the observed time-delays as the propagation time of these neutrons from the location of the lightning discharges.

Assuming a simple geometry consisting of a vertical lightning channel of length H equal to 2 km, at a distance r from the detector, the following approximate relation has been used to calculate the neutron yield per stroke

$$N = (N_D / \eta A) [(4\pi H r) / (\tan^{-1} H r)] (\exp \Sigma r) \quad (1)$$

where r (cm) = $13.8 \times 10^5 \sqrt{E_n}$, N is the total number of neutrons produced in the lightning channel, N_D the number of transmitted neutrons recorded by the neutron monitor, and η and A , the detection efficiency and the effective area of the monitor respectively. The total macroscopic cross-section, Σ , at the observation site, for neutrons of energy E_n (eV) is computed from the published microscopic scattering and absorption cross-section values⁷ for an atmospheric composition of 80% nitrogen, 20% oxygen.

Figure 2 shows the time-delay, t , and the number of neutrons recorded during the sampling time of 320 μ s for each of the 124 LT events. Evidently, 50 events have time delays in the range 10 – 50 μ s, which, if the constituent neutrons are assumed to be of 2.45-MeV energy, result in a total yield of 9×10^6 – 2×10^{10} neutrons per stroke. Such events would pass undetected in the measurements of Fleischer³, because of the average detection limit of 2.5×10^{10} neutrons per stroke inherent in his mode of detection. The time delays recorded for the remaining 65 events are well spread between 60 and 2×10^5 μ s and result in neutron yields too large to have gone undetected in earlier experiments. In addition, such high fluxes are also incompatible with the physical conditions obtained in a lightning channel and necessitate a reinterpretation of the large time delays exhibited by these events. It is possible that these events originated in multi-stroke lightning flashes; the first stroke triggers our system, but the neutrons are generated in a succeeding stroke belonging to the same or a different flash. Another plausible explanation is that these events comprise neutrons of lower energy produced⁸ other than by the postulated deuteron-deuteron fusion reaction, for example by $^{12}\text{C}(\text{H}, \text{n})^{13}\text{N}$ and $^{14}\text{N}(\text{H}, \text{n})^{15}\text{O}$. It is also worth considering that, when lightning strikes a tree, neutrons may be generated by the fusion of deuterons present in the vaporized

Table 1 Manually triggered (MT) and lightning-triggered (LT) events with different neutron numbers

No. of neutrons	No. of events	
	MT type	LT type
1	8,299 (98.84%)	10,818 (96.65%)
2	97 (1.11%)	250 (2.23%)
3	4 (0.05%)	40 (0.35%)
>3	0 (0.00%)	84 (0.77%)

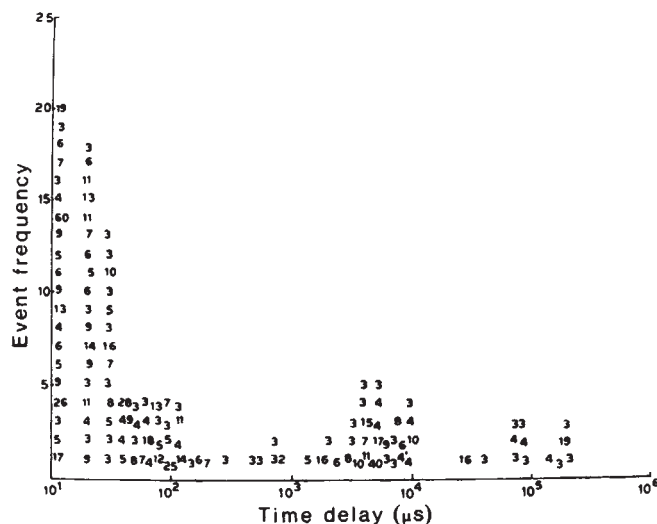


Fig. 2 The time-delay distribution of the 124 lightning-triggered (LT) events having ≥ 3 neutrons. The digits represent the number of constituent neutrons recorded in the sampling time of 320 μ s.

wood of the tree⁹. Such neutrons would undergo substantial energy degradation by the surrounding wood before reaching the detector and would be registered as events with large time delays.

The reason for considering these alternatives seriously becomes apparent when considering the following two events recorded at Gulmarg: a lightning is known to have struck a tree, 1.5 km away, resulting in the registering of 10 neutrons after a time delay of 30 μ s. Even after correcting for the delay in sensing the electric field variation due to its finite rise-time, the actual distance for this event is still underestimated by almost a factor of two when neutrons of energy equal to 2.45 MeV alone are considered. One may infer, therefore, that the neutron energy was > 2.45 MeV. On the other hand, the more plausible explanation for the small time delay is that the first detected neutron is a randomly-arriving cosmic-ray background neutron followed subsequently by neutrons from the lightning channel after a time lag.

In another instance, a fir tree, situated ~ 400 m away from our detector, was severely damaged in a lightning strike in which 33 neutrons were recorded in 320 μ s. The observed time delay of $7.174 \times 10^4 \mu$ s for this event places the source at a distance several orders of magnitude farther than the actual value of 400 m, assuming a neutron flux of 2.45-MeV energy. On the other hand, it is possible to account for the observed distance properly if we assume neutrons to have an energy as low as 0.2 eV. In fact, choosing neutrons with energies between 10^3 and 0.023 eV and accounting for the atmospheric absorption, we can reconcile the large time delays observed for 65 events with plausible yields of $3 \times 10^6 - 2 \times 10^{12}$ neutrons per stroke. We note that these calculations assume a continuous emission of neutrons along the lightning channel, which may not necessarily be true. Instead, neutrons may be generated in a limited length of the channel where conditions are most favourable. In the extreme situation of neutron generation from a point source, however, the calculated flux values do not change substantially from those of a line source. Furthermore, we must emphasize that the above estimates are only approximate and that, to calculate the neutron yield and interpret the observed time delay more accurately, one should consider atmospheric scattering and absorption effects under stormy conditions. Detailed modelling, using more accurate neutron propagation theories, is required for this purpose.

In conclusion, we have presented here evidence of possible neutron generation in lightning discharges, at yields of $\sim 10^7 - 10^{10}$ neutrons per stroke. That this is several orders of magnitude lower than the predicted values² is partly explained on the basis

that, while rescaling the exploding wire results to the lightning process, the significant differences in the respective plasma temperatures and reaction cross-section for the neutron generation were ignored. Further, it is not clear from our experiment whether these neutrons are thermonuclear in origin or are generated by non-thermal processes. In future, it will be important to obtain the lightning source distance independently to overcome the uncertainties, inherent in our technique, in estimating the yields from the recorded 'time delays'. There should also be provision for recording the lightning-generated neutron events simultaneously at another pile installed at an appropriate distance. A boron or cadmium cover placed over the pile would then further establish the genuineness of the events and broadly determine the energy of the neutrons.

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When does small-scale convection begin beneath oceanic lithosphere?

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Convection on a scale smaller than the horizontal dimensions of lithospheric plates can be produced by instabilities in the upper or lower boundary layer of the larger mantle flow. Small-scale convection associated with the upper boundary layer should produce a detectable gravity signal and affect the rate of cooling and subsidence of the lithosphere. I describe here a numerical model of small-scale convection in a fluid of variable viscosity. The results indicate that recently observed gravity anomalies¹ showing a pattern of highs and lows aligned in the direction of oceanic plate motion may be the result of small-scale mantle flow. The convective flow must begin in the first 6 Myr of lithospheric cooling to produce the observed signals, which is not inconsistent with constraints on the viscosity of the mantle. The calculated trend for the subsidence of the ocean floor is found to be almost linear with (time)^{1/2} even when small-scale convection has significantly changed the rate of subsidence. For average shallow asthenospheric viscosities of $\sim 10^{18}$ Pa s, the model subsidence can match data for the oceans^{2,3} and reproduce the magnitude and wavelength of the observed gravity anomalies.

The cooling of the oceanic lithosphere as it moves away from a ridge will be affected by small-scale convection. These effects are studied by calculating the flow in two-dimensional boxes induced by the cooling of a fluid from above. This two-dimensional treatment is justified by experiments on the interaction between two scales of convection, of which the smaller-scale flow is in the form of two-dimensional rolls⁴ where axes are oriented parallel to the larger flow, analogous to the plate

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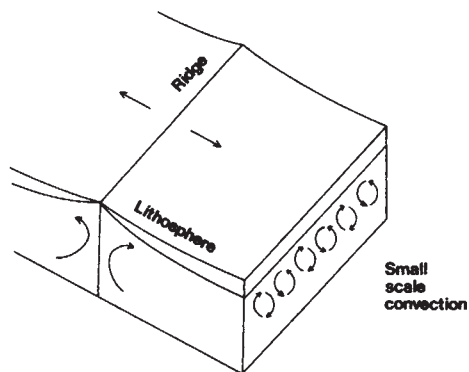


Fig. 1 This schematic diagram of the oceanic mantle shows the orientation expected for small-scale convection beneath the oceanic lithosphere. The end view, which shows a cross section of the small-scale rolls, is the plane in which the numerical calculations are done.

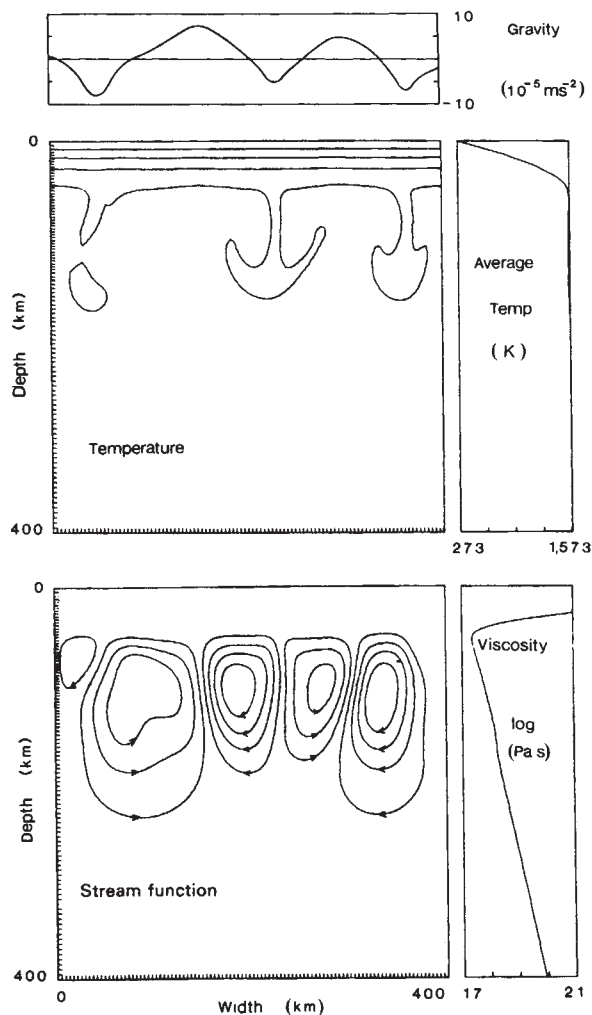


Fig. 2 The results of one numerical calculation of small-scale convection are shown for a box that represents a 400×400 -km region of the mantle after 10 Myr of cooling (5 Myr after the convection calculation was begun with an initial temperature profile from 5 Myr of conductive cooling). The positions of the 10^4 grid points used are indicated by the tick marks on the contour plots. A random temperature perturbation was given to each point at the start of the calculation. The dominant wavelength of flow has increased from a value of ~ 80 km after 2 Myr in the calculation to 150 km after 5 Myr. The temperature contours identify 600, 925, 1,250 and 1,573 K, in order from the top, and the streamfunction contours are evenly spaced between -1.04×10^{-4} and $8.5 \times 10^{-5} \text{ m}^2 \text{ s}^{-1}$. The parameter A , in equation (1), is set to give a viscosity of 10^{18} Pa s at 150 km and 1,573 K. The horizontally averaged temperature and viscosity are also shown, together with the model gravity anomaly that includes the effect of temperature variations and the effect of vertical deformations produced by the flow.

flow (Fig. 1). Here, the horizontal dimension is parallel to the ridge and the other dimension is the vertical; the model depth is 400 km and various widths are considered; time is proportional to distance from the ridge, assuming a constant plate velocity, which assumes, as previously⁵, that vertical gradients of velocity in the direction of plate motion are negligible, which should be valid over several hundred kilometres and makes these calculations tractable.

The two-dimensional Navier-Stokes equations of energy, mass and momentum conservation in a fluid of variable viscosity and infinite Prandtl number⁶ are solved using an explicit finite difference formulation with grids of variable spacing to allow a fine mesh in the region of high gradients of viscosity⁷. The boundary conditions make the side and bottom insulating and stress-free; the boundary conditions at the top are a constant temperature (273 K) and a flow that entails no slip. The starting temperature profile is that for 5-Myr conductive cooling of a half-space, initially at 1,573 K, and an adiabatic gradient (0.3 K km^{-1}) is added when calculating the viscosity. Two types of temperature perturbations are used to induce flow: when studying the onset and growth of convection cells, a perturbation of random magnitude between 0 and 1 K is given to each point; when the effects of one convective wavelength are considered, a periodic perturbation is used.

The values of constants in the Navier-Stokes equations are: the temperature scale, 1,300 K; thermal expansion coefficient, $\alpha = 4.0 \times 10^{-5} \text{ K}^{-1}$; thermal diffusivity, $\kappa = 1.0 \times 10^{-6} \text{ m}^2 \text{ s}^{-1}$, and the acceleration of gravity, $g = 1.0 \times 10^1 \text{ m s}^{-2}$. Viscosity, ν , is taken to be a function of temperature, T , and pressure, P , as⁸

$$\nu(T, P) = A \exp[(E + PV)/RT] \quad (1)$$

where A is a constant that determines the average level of viscosity and R is the universal gas constant. The activation energy, E for creep in olivine (measured range⁹, 300–500 kJ mol^{-1}) controls the temperature dependence of viscosity; the activation volume, V , determines the pressure dependence of viscosity and has been found to be in the range $(1.0\text{--}2.0) \times 10^{-5} \text{ m}^3 \text{ mol}^{-1}$ based on experimental and theoretical estimates¹⁰. Many model cases have been calculated to assess the importance of the parameters that determine viscosity (W.R.B. and E.M. Parmentier, in preparation), but the results of only one case are discussed here. For this we take $E = 420 \text{ kJ mol}^{-1}$ and $V = 10^{-5} \text{ m}^3 \text{ mol}^{-1}$ and use a value of A that gives a viscosity of 10^{18} Pa s at 150-km depth at the initial temperature in the model. The activation volume is critical in reconciling different estimates of mantle viscosity. An average mantle viscosity of $\sim 10^{21} \text{ Pa s}$ is required by post-glacial rebound rates¹¹, but several geophysical observations require lower viscosity ($< 10^{19} \text{ Pa s}$) at shallow depths in the mantle (~ 100 km) under the oceanic lithosphere and under tectonically active areas of the con-

tinents^{12–14}, which is accomplished by the pressure dependent term, PV .

Figure 2 shows characteristic features of results in terms of contours of temperature and stream function for a time 5 Myr from the origin of the calculation at time 5 Myr, the point from which the temperature perturbations grow. This means that the cooling time for onset of convection is < 5 Myr. The area where the isotherms are almost horizontal is the conductive lid where the viscosity is high enough to preclude flow. The advective transfer of heat to the lid, cold material flowing away from the lid is replaced by hotter material, must have an effect on the rate of cooling of the lid. To quantify this, we calculate the average temperature drop, $\Delta T_L(t)$, at time t , in the top portion of the box to a depth, Z_L , here taken to be 150 km. To relate

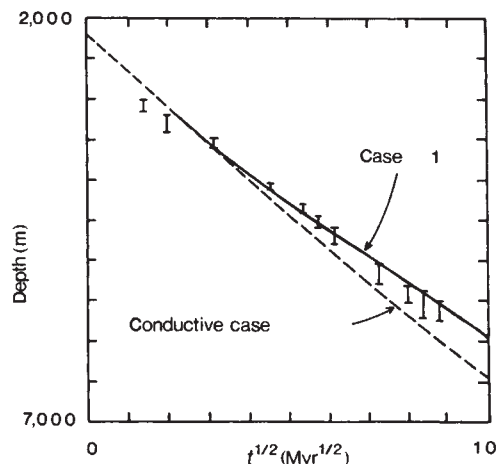


Fig. 3 The subsidence for case 1 calculated with $\alpha = 3.8 \times 10^{-6} \text{ K}^{-1}$ in equation (2) plotted against $t^{1/2}$. A ridge crest depth of 2,200 m is assumed in the calculation of subsidence. Also shown is the ocean depth data for the Pacific² out to 100-Myr age. For reference the subsidence for purely conductive cooling calculated with the same thermal expansion, α , and thermal diffusivity, κ , is shown.

ΔT_L to water-loaded thermal subsidence of the lithosphere, S , we use the relationship

$$S(t) = \alpha \frac{\rho_m}{(\rho_m - \rho_w)} \Delta T_L Z_L \quad (2)$$

where $\rho_m = 3.3 \times 10^3 \text{ kg m}^{-2}$ and $\rho_w = 1.0 \times 10^3 \text{ kg m}^{-2}$ are the densities of the mantle and water respectively. This assumes that Z_L is the level of isostatic compensation where horizontal pressure gradients are negligible. Thus, by assuming different thermal expansion coefficients, α , for the convective and conductive cases, each can have the same slope on a plot of S against $t^{1/2}$. Such a plot, using data on ocean floor subsidence or depth is linear for the first 80 Myr of cooling^{2,3}, which has been considered to reflect only conductive cooling of the lithosphere³ or cooling with a phase change at the base of the lithosphere¹⁵. Although the data on ocean depths for ages of $< \sim 80$ Myr are consistent with conductive cooling of the lithosphere, with $\kappa = 1.0 \times 10^{-6} \text{ m}^2 \text{ s}^{-1}$ and $\alpha = 3.2 \times 10^{-5} \text{ K}^{-1}$, they can be fit equally well by the results of the convection calculation, if $\alpha = 3.8 \times 10^{-5} \text{ K}^{-1}$, as shown in Fig. 3, or by a range of results of convection calculations for different viscosities. The higher value for α is in better agreement with the laboratory estimates of α for olivine in the temperature range appropriate to this problem¹⁶. If different values for Z_L are chosen, then different values of κ and α are required to match the subsidence data, but the major conclusions remain unchanged.

If small-scale convection begins in the first few million years of lithospheric cooling, then the flattening of the plot of ocean depth against $t^{1/2}$, inferred to occur at ~ 80 Myr, cannot be caused by the onset of such convection, as suggested previously^{5,17}. Another view of the data is that the flattening is gradual and the result of heat transported from deeper in the mantle, with mantle plumes as possible carriers of this heat¹⁸. In any case, the main result of these calculations is that when small-scale convection begins under the cooling lithosphere, a sudden change in the rate of subsidence does not occur.

The Seasat oceanic altimetry data include features that may indicate when vigorous small-scale convection begins. Haxby and Weissel¹ have noted linear gravity anomalies of 4–10 mgal amplitude with a wavelength of 150–220 km (see Fig. 4), approximately parallel to the direction of plate motion, the orientation expected for this type of convection⁴. The anomalies are seen over regions of the lithosphere as young as 6 Myr in age, and older. Here the wavelengths of the flow depend on the viscosity parameters assumed in equation (1), but the magnitude

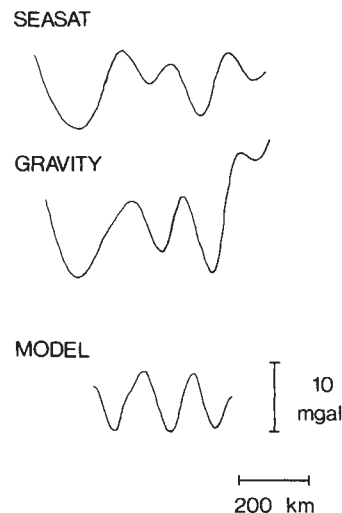


Fig. 4 Seasat-derived gravity anomalies and filtered shipboard gravity anomalies for profiles that are perpendicular to the trend of linear gravity highs and lows observed in the Seasat data¹, together with a model gravity signal. The profiles¹ are centred on 0° N latitude and 145° W longitude and are oriented roughly N–S. The shipboard gravity has been filtered to remove signals with frequencies too high to be detected by Seasat. The model example is for the same time as the case illustrated in Fig. 1 and shows that the calculations predict gravity anomalies in the same magnitude and wavelength range as those observed.

of the predicted signals are relatively insensitive to the average viscosity. The gravity anomalies produced in the calculation by convection have been estimated using a standard method¹⁹ and assuming local isostasy. The wavelength and magnitude of the anomalies increase with time, but as shown in Fig. 4, small-scale convection can produce anomalies with the observed amplitude and wavelength. The flexural rigidity of the lithosphere will diminish the magnitude of the signal, but for young regions this effect should be small. Because the gravity signals are produced by the topography that results from convective stresses, they can be 'frozen in' as the lithosphere cools and its flexural rigidity increases.

Thus, small-scale convection organized into vigorous rolls by a lithospheric age of 6 Myr could produce the observed gravity features. This requires asthenospheric viscosities at least as low as those considered here ($\sim 10^{18} \text{ Pa s}$). But average viscosities more than a factor of 10 lower than this predict a subsidence that cannot match the oceanic data without assuming extreme values for the thermal expansion and diffusivity of the lithosphere.

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Sulphur flows at Lastarria volcano in the North Chilean Andes

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Although several authors have suggested that sulphur plays an important role in the volcanism on the jovian moon Io¹⁻⁸, some deny the participation of elemental sulphur there, at least in the form of cooled flows⁹. Studies of volcanism on Io have focused attention on the role of sulphur in terrestrial volcanism. On Earth, fumarolic sulphur is very common in almost all active volcanoes, but sulphur flows are rare, having been reported only on Siretoko-Iōsan volcano in Japan¹⁰, on Sierra Negra and Azufre volcanoes in the Galápagos Islands^{11,12} and on Mauna Loa in Hawaii^{13,14}. Of these, the Siretoko-Iōsan example is the only natural molten sulphur flow to have been observed in the process of formation; the others being rather poorly exposed. I describe here a singularly well-exposed example of sulphur lava flows, on Lastarria volcano in Chile, where the liquid sulphur appears to have been formed by melting of fumarolic sulphur deposits caused by a rise in the thermal gradient owing to the renewed activity of the magmatic source.

Lastarria is a small compound andesite-dacite volcano rising from ~4,200 to 5,700 m in the Antofagasta Region of northern Chile. Although there is no recorded history of eruptions, fumaroles are conspicuously active on the northwestern flank and at the summit; prominent sulphur flows have been identified below the northwesternmost fumarole and minor flows are present near a summit crater. Fumaroles are located on the edges and flanks of the younger northernmost craters (Fig. 1). Fumarolic vents are either fractures (~1 m × 50 cm) in andesite, or chimney-like (15 cm in height, 7 cm in diameter) and cone-like 'hornitos'¹¹ built on a base of unconsolidated pyroclastic deposits. Fumarolic gases have also percolated to the surface over wide areas in the fumarolic fields (>2,500 m²) and vent to the surface through random cracks and fissures.

Sublimated sulphur has a deep yellow-orange colour on the internal moist parts of the brittle walls of the active chimneys; the external powdery surface is light yellow. Sulphur sublimated at the chimney is cooled quickly and becomes crystalline (orthorhombic) with a stable light yellow colour. Around the higher temperature parts of the fracture vents, the altered rock is black, grading into a brown-orange and then into an orange-yellow powdery sulphur deposit a few centimetres away. The brown-orange sulphur, although solid where accessible, retains a liquid morphology with flow droplets which have rounded margins (2 cm long, 0.5–1 cm wide, 0.5 m thick). This material grades inwards to masses of dark red amorphous sulphur with a glassy lustre filling cavities and coating internal fracture surfaces of the extensively altered and fractured outer crust (1–5 cm) of the host rock. These colours may imply that the sulphur has reached a temperature hotter than 250 °C¹⁵.

Two main flows of sulphur (sloping at 24–28°) have been identified on the middle of the north-west flank of Lastarria (Fig. 1). The distal end of the lower flow (350 m long) underlies a pyroclastic flow which itself is covered by a younger higher sulphur flow (220 m long). The recurrence of sulphur flows through time is also demonstrated by the presence of different flow units (at least five within the lower flow) showing complete cooling and a varying degree of morphological degradation between units, that is, the lowest and oldest flow has lost its surface structures, probably by wind, snow and water action. There is no evidence bearing on the absolute age of the sulphur flows, but it is clear that they are not new.

The thickness of individual flow units decreases downslope from 20 cm in proximal areas to 10 cm at the distal front and

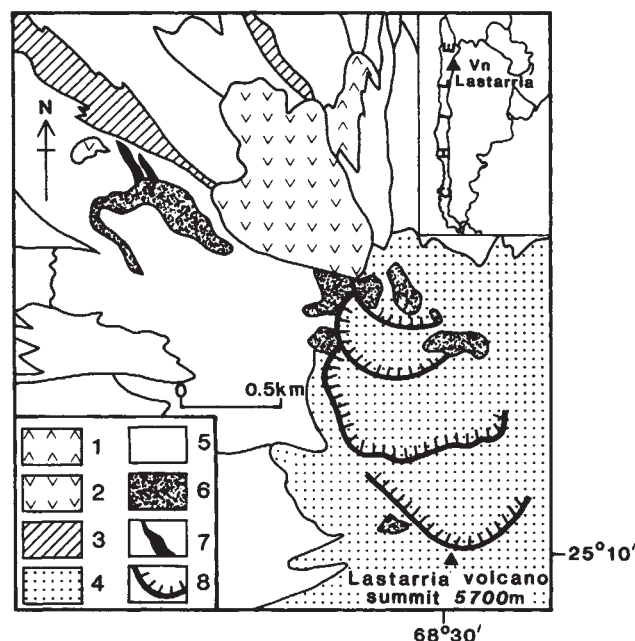


Fig. 1 Map of the northwestern part of Lastarria volcano: (1) lava flows; (2) dome; (3) 'hot avalanche' deposits; (4) ash fall deposits; (5) pyroclastic flow deposits; (6) fumarolic deposits; (7) main sulphur flows; (8) craters.

flow widths vary irregularly between 1 and 2.5 m. Structures and surface textures resemble those of pahoehoe lava flows. Channels of 50–80-cm width and levées up to 10 cm high are common; cascade-lobes (1.5 m long) have flowed down from levée edges and successive flow units (pulses) are confined to previous channels until they spill over the levées (Fig. 2a and b). Pahoehoe-like surface texture is always present, with concentric and twisted ridges (2–3 cm high) oriented perpendicular to the flow directions. Some of these ridges have folded over to form an imbricated scaly surface. These structures show clearly that the flows cooled more rapidly at the margins than at the central parts, thereby forming a surface crust.

Near the sources, sulphur flow colour is a pale greenish-yellow, with grey tone at the distal ends. Incorporated fragmental constituents, comprising up to 28% by volume, are composed of altered grains of bleached clayey rock (2–5 mm) and pyroclastic material (0.5–5 cm) distributed without layering within the flow, suggesting laminar emplacement. Very irregularly shaped vesicles (1–5 mm) in the upper part (up to 3 cm thick) of individual flows have been observed, reaching up to 26% in volume. At the base, a thin layer (<5 mm) of yellow sulphur penetrates as small lobes into the loose tephra over which the flow passed. Confined to the neighbourhood of the fumaroles, smaller flows (~2 × 3 m, 'mantle flows') of deep yellow sulphur 2–5 cm thick have been identified, their surface texture is pahoehoe-like with complex wrinkled ridges exhibiting wavelengths of 0.5–2 cm (Fig. 2c). Less common flows have smooth surfaces and are formed by a series of overlapping toe-like lobes (1.5 cm wide), visible throughout the thickness (0.5–2 cm) of the flows. The former flows have developed a skin of amorphous cooled yellow sulphur. Isolated spatter droplets (<1 cm thick, 20 cm long, 1–5 cm wide) of yellow sulphur, perhaps splashed out of the source, have also been observed. The total volume of sulphur in all observed flows does not exceed 300 m³.

These different sulphur flows have emerged from the surrounding fractured and altered rocks, heavily coated by fumarolic sulphur deposits, and no specific vents could be identified. Flow heads are located either on sloping surfaces or on the top of a slope. Fumarolic deposits are partially covered by pyroclastic material and clearly existed before some of the explosive eruptions took place. The intercalation of a pyroclastic flow between main sulphur flows suggests the presence of a

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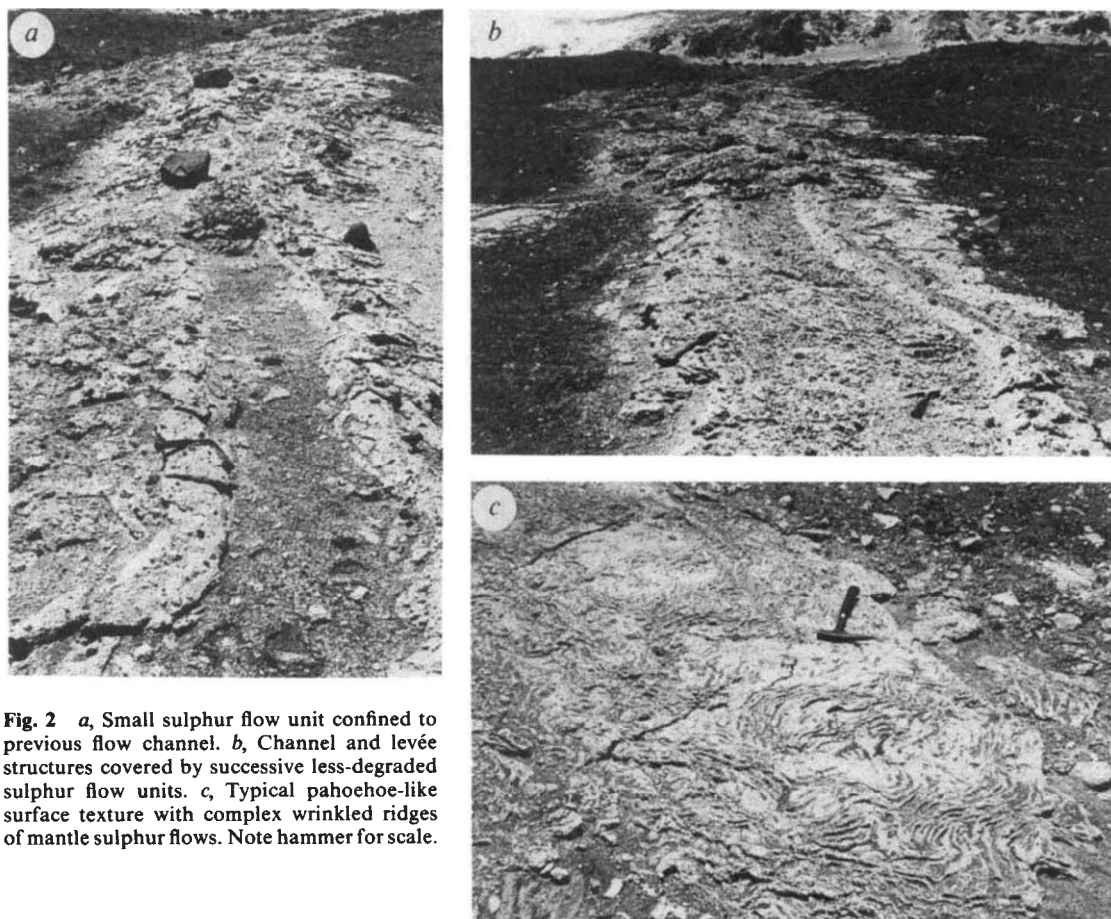


Fig. 2 *a*, Small sulphur flow unit confined to previous flow channel. *b*, Channel and levée structures covered by successive less-degraded sulphur flow units. *c*, Typical pahoehoe-like surface texture with complex wrinkled ridges of mantle sulphur flows. Note hammer for scale.

fresh magmatic source that provided a rise in the local thermal gradient necessary for melt and mobilization of sulphur deposits. Subsequently, liquid sulphur reservoirs could have been formed, or the volcano slope could have allowed melted sulphur to flow through fractured rocks until it reached the surface, analogous to the process that has been postulated¹⁴ for the Mauna Loa sulphur flow.

The temperature of formation may be tentatively inferred from the mode of deposition and the colour retained. Bright yellow-orange sublimated (fumarolic) sulphur was deposited between the boiling point of water (83–84 °C) (no water vapour was condensed on the inner parts of the vents) and ~113 °C, the melting point of sulphur. Brown and red liquid-morphology sulphur deposited near fracture vents was probably formed at temperatures above 250 °C, as at high temperature liquid sulphur becomes extremely reactive and the colour is obscured by irreversible darkening due to organic impurities¹⁵. The yellow reverted colours indicate that the sulphur flows cannot have cooled from above 250 °C¹⁵ and it is unlikely that they were mobilized above 160° because of the resulting very high viscosity (Fig. 3); the temperature must therefore have been between 113 and 160 °C, comparable to the proposed¹⁰ eruptive temperatures of ~130–160 °C for Siretoko-Iōsan flows based on measurements away from the vent.

Liquid sulphur has unusual rheological properties (Fig. 3). Cooling Lastarria sulphur flows below 160 °C would have produced rheological flow profiles similar to those of silicate lava, with a stiff crust enclosing a fluid interior. Theoretical models and experimental studies which interpret the role of sulphur in eruptive processes on Io^{8,14,16–18} have not considered natural conditions in sulphur flow formation on Earth. It is important to establish whether these sulphur flows behaved as Newtonian, Bingham or even as pseudoplastic fluids. Experiments have indicated that pure liquid sulphur behaves as a Newtonian fluid^{19,20}, but homogeneously-dispersed accidental fragments (28%) and gas bubbles in Lastarria sulphur flows could sig-

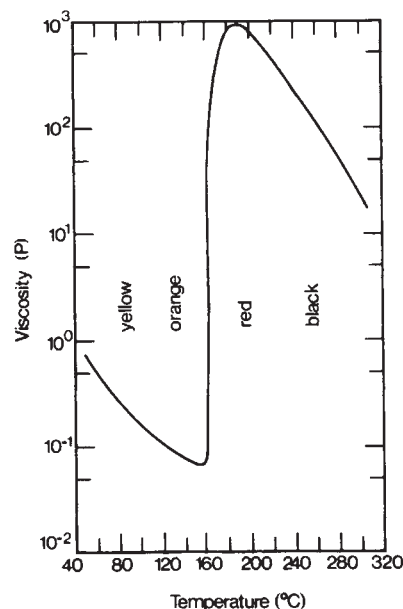


Fig. 3 The variation of viscosity²⁶ and colour of liquid sulphur with temperature.

nificantly change this behaviour to resemble those silicate melts that behave as non-Newtonian (even pseudoplastic) fluids owing to the presence of crystals and gas bubbles²¹.

The Siretoko-Iōsan¹⁰ and Lastarria sulphur flows are the only flows reported to have regularly-spaced surface ridges. Three conditions must be satisfied to develop surface folding in a viscous newtonian fluid: high contrast between surface and interior viscosities, large compressive forces relative to the

weight of the fluid and surface viscosities low enough to allow deformation without fracturing²². The resulting fold wavelengths are proportional to the crustal thickness²³. This can be expressed²³ as $Ld/H \geq 28/\ln R$, where Ld is the arc length of the ridge, H is the thickness of the crust of variable viscosity and R is the ratio of surface to interior viscosities. However, for sulphur flows between 113 and 160 °C, the viscosity contrast is ≤ 2 (Fig. 3), which is insufficient to produce ridges of the magnitude described above, given the ~3-cm thickness of the crust (vesicular layer). On the other hand, Hulme's^{24,25} model for the yield strength (Bingham fluids) and effusion rate of lava flows, based on their morphology, is also inapplicable to the Lastarria sulphur flows, predicting absurdly high (1,800–3,300 m³ s⁻¹) effusion rates.

Further study of the flows at Lastarria and their structures could help in understanding the emplacement of sulphur flows. All reported natural terrestrial sulphur flows seem to have been formed by remobilization of fumarolic deposits, probably at the low viscosity-temperature stage, although small amounts of highly viscous molten sulphur could also be present. Rheological properties of sulphur flows could be modified by incorporation of accidental fragments and by formation of vesicles, which, by lowering the local density, could either favour or disrupt an internal temperature-viscosity layering, and modify surface folding, of the flow¹⁸. Such aspects should also be considered in speculations about volcanism on Io.

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Oxygen isotope ratios in N₂O from different environments

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Nitrous oxide (N₂O) is an important 'greenhouse' gas (besides CO₂ and CH₄) and is also involved in the destruction of ozone in the stratosphere. The relative importance of major sources and sinks of N₂O for a global budget has yet to be assessed accurately. Measurements of the ¹⁵N/¹⁴N (refs 1, 2) and ¹⁸O/¹⁶O isotopic composition of N₂O from different sources and the comparison with the isotopic composition of atmospheric N₂O could lead to more accurate estimates of the contributions to the atmospheric pool by the different production mechanisms. Here we report the first results for ¹⁸O/¹⁶O ratios in N₂O collected from different environments, measured by high-resolution infrared spectroscopy using tunable diode lasers. The results show a considerable range in $\delta^{18}\text{O}$ (Table 1), which could eventually lead to an isotopic budget for atmospheric N₂O.

Nitrous oxide is produced by bacterial processes of nitrification and denitrification mainly in soils and oceans^{3–7}, and to some extent during fossil fuel combustion^{8–10}. Photochemical destruction occurs in the stratosphere^{11–15}. Recent increases in the atmospheric concentration of N₂O^{8–10} are attributed to increased fossil fuel combustion and fertilizer use.

A global budget for N₂O has not yet been established accurately. Quantitative estimates of the production and destruction rates of different sources and sinks and of the atmospheric residence time scatter considerably^{4,16}.

More information may be obtained from stable isotope data on nitrogen and oxygen in N₂O. The isotopic composition of N₂O from different environments is expected to vary because of different formation processes, specific equilibrium fractionation and chemical and biological kinetic isotope effects. If this is indeed so, then more accurate estimates could be obtained for a global N₂O budget from the comparison with the isotopic composition of atmospheric N₂O.

Recently ¹⁵N/¹⁴N data were reported for N₂O (refs 1, 2), revealing small differences between oceanic and atmospheric

Table 1 Concentrations and $\delta^{18}\text{O}$ for N₂O from different environments

Sample	N ₂ O concentration (p.p.m.)	$\delta^{18}\text{O}$ (SMOW) (‰)
Ambient air (Albany, New York)		
Dec. 1981		42.7 ± 0.6
May 1982	0.30–0.35	47.2 ± 0.4
Sept. 1982		45.3 ± 0.3
Feb. 1984		45.6 ± 0.4
March 1984		43.4 ± 0.2
March 1984		48.3 ± 0.9
Green Lake, New York, water		
Sept. 1982, 13 m depth	3.5–4.1	93.0 ± 0.3
Soil gas		
Sept. 1983	0.57	35.9 ± 0.4
Stackgas from coal-burning power plant Binghamton, New York		
Sample 1	40.6	58.6 ± 0.4
Sample 2	50.0	59.9 ± 0.4
Car exhaust	10.1	46.9 ± 0.3
N ₂ O tank 1		34.1 ± 0.2*
N ₂ O tank 2		37.0 ± 0.3

* Measured by isotope ratio mass spectrometry (R. Lawrence and J. White, Lamont-Doherty Geological Observatory).

N₂O. Based on simple mass considerations the oxygen isotope effects are expected to be larger. As oxygen is involved in oxidation and reduction it may be reasonable to expect larger variations in $\delta^{18}\text{O}$ of N₂O than in $\delta^{15}\text{N}$, and thus more easily discernible information may be obtained from the measurements of $\delta^{18}\text{O}$ in N₂O ($\delta^{18}\text{O}$ (‰) = $\{[(^{18}\text{O}/^{16}\text{O})_{\text{sample}}]/(^{18}\text{O}/^{16}\text{O})_{\text{SMOW}}\} - 1\} \times 10^3$, where SMOW is standard mean ocean water). Oxygen-18 exchange with water of precursor and intermediate oxygen-bearing nitrogen compounds seems to be negligible¹⁷ and the isotopic identity of dissolved and atmospheric N₂O is preserved.

The ¹⁸O/¹⁶O isotope ratios were measured by high-resolution infrared spectroscopy using tunable diode lasers. This technique is free from possible interference by trace CO₂ contamination. The measurements are performed on two neighbouring vibra-

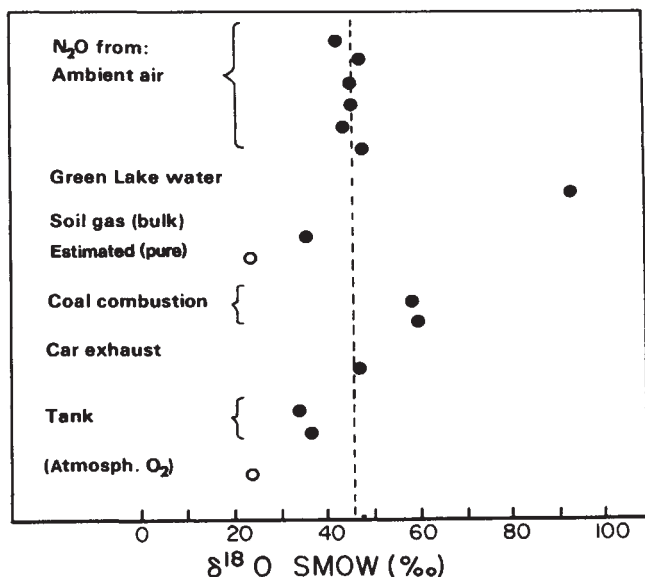


Fig. 1 $\delta^{18}\text{O}$ (SMOW) in N_2O from different environments and sources. Also shown is $\delta^{18}\text{O}$ for atmospheric oxygen²³.

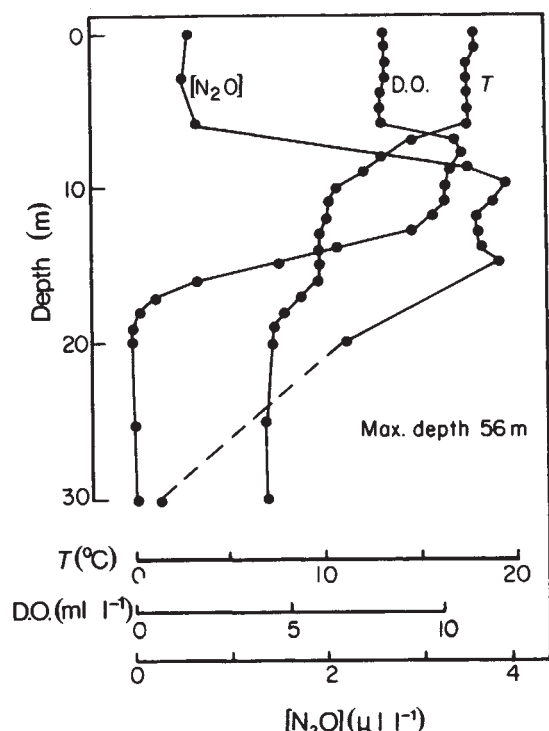


Fig. 2 Profiles for temperature (T), dissolved oxygen (D.O.) and $[\text{N}_2\text{O}]$ from the deep section of Fayetteville Green Lake, New York ($43^\circ 03' 00''\text{N}$, $75^\circ 47' 48''\text{W}$).

tion-rotation absorption lines for N_2^{18}O and N_2^{16}O , namely, P(27) and P(64) or P(26) and P(63) of the ν_1 band at $1,225\text{ cm}^{-1}$. These line pairs are chosen because about equal absorbance is obtained for the two molecular species at their natural abundance. The laser frequency is modulated by a current ramp to scan the two absorption lines and alternatively passed through two parallel absorption cells of 40-cm length. Whereas one cell contains reference N_2O at all times, the other cell is filled alternatively with sample, reference and standard N_2O . A small sinusoidal current variation is superimposed on the ramp to produce the optical derivative signal at the HgCdTe detector, which is analysed synchronously with a lock-in amplifier. The rectified lock-in output is sampled with a signal

averager. Several hundred scans each, from sample and reference cells, are accumulated for a single measurement with signal to noise levels typically $> 10^4$. The $\delta^{18}\text{O}$ of the sample is then computed from the integrated peak ratios. Minimum sample size at present is $\sim 0.6\text{-cm}^3$ N_2O (STP). As reference gas we used commercially available tank N_2O , which is manufactured by dehydration of synthetic ammonium nitrate. Oxygen-18 calibration was obtained from N_2O enriched in ^{18}O . For the conditions used, the system response is linear in $\delta^{18}\text{O}$. Calibration against the SMOW scale was obtained by converting quantitatively the tank N_2O to H_2O with H_2 and Cu; $\delta^{18}\text{O}$ (SMOW) was determined by isotope ratio mass spectrometry. This technique is also applicable to the nitrogen isotopes, with the capability of measuring $^{15}\text{N}/^{14}\text{N}$ for both N atoms individually.

Air samples (ambient air, soil gas, exhaust gases from a coal-fired power plant and an automotive engine) were collected into tanks with a compressor. N_2O concentrations in the samples were determined by gas chromatography with an electron-capture detector¹⁸ for small concentrations and with a thermal conductivity detector for large concentrations. The large volume samples were then passed, in the laboratory, at a low flow rate through a dry ice-acetone cold trap and drierite and ascarite columns for removal of H_2O and CO_2 ; the N_2O was trapped at a pressure of 100 torr at 77 K in a large glass coil, purified by gas chromatography and measured as described above. Soil gas samples were collected from under a sealed plastic sheet covering an area of 100 m^2 . Water samples were first stripped with counterflowing He and the N_2O separated from the collected dissolved gases. No significant isotope fractionation was observed when tank N_2O , diluted to ambient air concentration by pure N_2 evaporated from liquid nitrogen, was subjected to the same separation procedure.

The types of samples analysed and the results are listed in Table 1. The $\delta^{18}\text{O}$ values and uncertainties are means and standard deviations from multiple measurements and do not contain any systematic error from the enriched N_2^{18}O standards. The $\delta^{18}\text{O}$ results are also shown in Fig. 1. A fairly wide range of $\delta^{18}\text{O}$ was obtained from this limited set of samples. The six samples of ambient surface air taken at different locations yield an average $\delta^{18}\text{O}$ of $45.4 \pm 1.0\text{‰}$. More data are needed to investigate the observed scatter which may be due to sampling location or time, or both.

Dissolved N_2O from Green Lake, New York, shows the highest $\delta^{18}\text{O}$. This sample, although insignificant for global production, is from a special freshwater environment with conditions similar to a fjord. Green Lake is meromictic and the water sampled at a depth of 13 m was from below the chemocline. Profiles of temperature, dissolved oxygen and N_2O concentrations at the time of sampling are shown in Fig. 2. There is an extended layer where peak N_2O concentrations ranged 3.5–4.1 p.p.m. The data suggest that the N_2O is derived from denitrification, although production by nitrification cannot be totally excluded. In the process of bacterial N_2O consumption the lighter molecule will be reduced preferentially, leaving behind N_2O enriched in ^{18}O . In fact, we have measured a substantial ^{18}O isotope effect in the reduction of N_2O by *Pseudomonas aeruginosa* (unpublished data). From a series of experiments with the remaining unreacted substrate N_2O fraction, f , ranging from 55 to 10%, the per mil enrichment factor

$$\epsilon = 10^3 \ln \left(\frac{10^{-3}\delta + 1}{10^{-3}\delta_0 + 1} \right) / \ln f \quad (1)$$

(where δ_0 is the original substrate $\delta^{18}\text{O}$), was found to be -42‰ for $T = 10^\circ\text{C}$ and -37‰ for $T = 26^\circ\text{C}$. These values are similar, but expectedly larger than those for ^{15}N (ref. 19), although experimental conditions were not identical. Such a mechanism could be responsible for the high $\delta^{18}\text{O}$ in N_2O from the Green Lake waters.

N_2O from the bulk soil gas sample collected from a field heavily fertilized with manure shows a $\delta^{18}\text{O}$ value below that of atmospheric N_2O . The N_2O concentration under the plastic

sheet increased from 0.35 to 1.10 p.p.m. over a period of 6 h as a result of soil gas mixing with overlying air. We suspect that this N_2O is predominantly from nitrification. N_2O from nitrification is a byproduct from reduction of nitrite²⁰; it has been shown that the oxygen in the intermediate nitrite is derived equally from molecular O_2 and H_2O ^{21,22}. From the oxygen isotopic composition of atmospheric oxygen and of precipitation and ground water a relatively light N_2^{18}O may, therefore, be expected. The isotopic composition of the 'pure' soil N_2O component can be estimated from the result of the analysed bulk sample ($\delta^{18}\text{O} = 35.9\%$), the excess N_2O and $\delta^{18}\text{O} = 45.4\%$ for atmospheric N_2O . The result is $\delta^{18}\text{O} = 24\%$ for this soil derived N_2O , as indicated in Fig. 1, where, for comparison, $\delta^{18}\text{O} = 23.5\%$ for atmospheric oxygen is also shown²³.

Production of N_2O by fossil fuel combustion is estimated to be a small fraction of the global N_2O production^{4,8-10}, but N_2O from biomass burning may be substantial²⁴. The N_2O concentrations measured in our samples from coal combustion and from automobile exhaust (from a test engine equipped with catalytic converter²⁵) are similar to those reported by others⁸⁻¹⁰, who have also proposed formation mechanisms. The $\delta^{18}\text{O}$ of N_2O in these gases are rather large considering the possible sources of combustion oxygen, which may indicate that N_2O is an intermediate reaction product and that efficient N_2O decomposition is also occurring during combustion.

Oxygen exchange between N_2O and water was found to be extremely small. A volume (40 cm^3) of tank N_2O (STP) was stored in a vacuum container together with 1.0 g of H_2^{18}O (99 atom %) at room temperature for 1.85 yr, during which we observed an increase of $4 \pm 1\%$ above the initial $\delta^{18}\text{O}$ in the N_2O , which allows us to estimate a rate constant. If this is applied to the conditions in the ocean and atmosphere, it becomes obvious that isotopic exchange is negligible for even the longest residence time estimates^{3,26}.

The observed range in $\delta^{18}\text{O}$ for N_2O supports the view that the $\delta^{18}\text{O}$ values are regulated primarily by the mechanism of N_2O formation in different environments and reservoirs. For N_2O from nitrification it is conceivable that $\delta^{18}\text{O}$ is largely determined by the isotopic composition of the available molecular O_2 and H_2O , but also influenced by kinetic effects. Thus, N_2O from nitrification in soils and oceans may have different $\delta^{18}\text{O}$, owing to isotopic differences in the available O_2 and H_2O ; particularly if oceanic N_2O is produced mainly in oxygen deficient layers, where the dissolved oxygen is enriched in ^{18}O compared with atmospheric oxygen²³. In the case of denitrification, where N_2O is an intermediate of nitrate reduction, the oxygen of N_2O is derived from nitrate-oxygen. As both nitrate-oxygen and nitrite-oxygen do not readily exchange with water-oxygen¹⁷, the isotopic composition of nitrate-oxygen (itself a product of nitrification), besides kinetic effects, may determine $\delta^{18}\text{O}$ of N_2O . Also remnant N_2O from partial N_2O reduction could be substantially enriched in ^{18}O .

We are currently enlarging our database, especially for atmospheric N_2O and soil gas samples from different soil ecosystems. Also included will be N_2O samples from the minimum oxygen layers of the Atlantic Ocean and N_2O sampled from the stratosphere, to test whether an isotopic N_2O budget can be obtained.

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Tellurium species in seawater

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The marine geochemistry of tellurium is unknown, being one of the few elements whose concentrations in seawater have never been reported^{1,2}. The major problem in studying Te stems from its rarity and the poor sensitivities of most analytical methods (even its crustal abundance is still controversial³). We have developed a very sensitive method allowing accurate measurements to be made of Te(IV), Te(VI) and total Te in seawater and we present here several vertical profiles of these species, which were obtained from the ocean. The Te profiles resemble in part the behaviour of Se and in part that of Po, reflecting the position of Te between Se and Po in Group VIb of the Periodic Table. Te, like Se, is found to be present in two oxidation states, Te(VI) and Te(IV), but their relative stabilities are reversed with respect to Se⁴⁻⁷. Se(VI), which is thermodynamically stable, and the unstable Se(IV) both exhibit nutrient-type distributions, but the distribution of Te, like ^{210}Po ⁸, shows evidence of strong scavenging by marine particulates, consistent with the chemical speciation of Te(VI) as a hydrosilicate ($\text{Te}(\text{OH})_6$), similar to $\text{Po}(\text{IV})(\text{OH})_4$, rather than as an oxyanion, like $\text{Se}(\text{VI})\text{O}_4^{2-}$.

Water samples were obtained either by Niskin bottles with Teflon-coated internal springs hung on steel wire (Panama Basin) or by rosette-mounted Niskin bottles (Long Lines 23 and TTO 241). The samples were acidified to $\text{pH} < 2$ by adding 4 ml of 6N HCl per litre of seawater and then stored in acid-cleaned polyethylene bottles, under which conditions no changes in concentration and speciation were observed over one year.

The analyses were made by graphite-furnace atomic absorption spectrometry coupled with H_2Te generation. Te(IV) was reacted with NaBH_4 to form H_2Te , which was subsequently stripped out of solution with Ar gas, trapped *in situ* in the graphite furnace of an atomic absorption spectrometer and then atomized (a method similar to one used previously for bismuth⁹). As the concentrations of Te species in ocean water are extremely low, alkaline coprecipitation was used before the H_2Te generation. For Te(VI) and total Te measurements, Te(VI) had to be reduced first to Te(IV) by boiling in hydrochloric acid, as only Te(IV) reacts with NaBH_4 . Details of the method will be given elsewhere.

The total concentration of Te and those of its individual oxidation states have been measured at two stations, Long Lines 23 (LL 23) in the Angola Basin of the eastern South Atlantic and Endeavor 108 in the Panama Basin; in addition, total Te has been measured at TTO 241 in the western North Atlantic.

At LL 23 total Te decreases smoothly from a surface maximum of 1.30 pM to ~0.6 pM at 2,500 m (Fig. 1a), but below this the

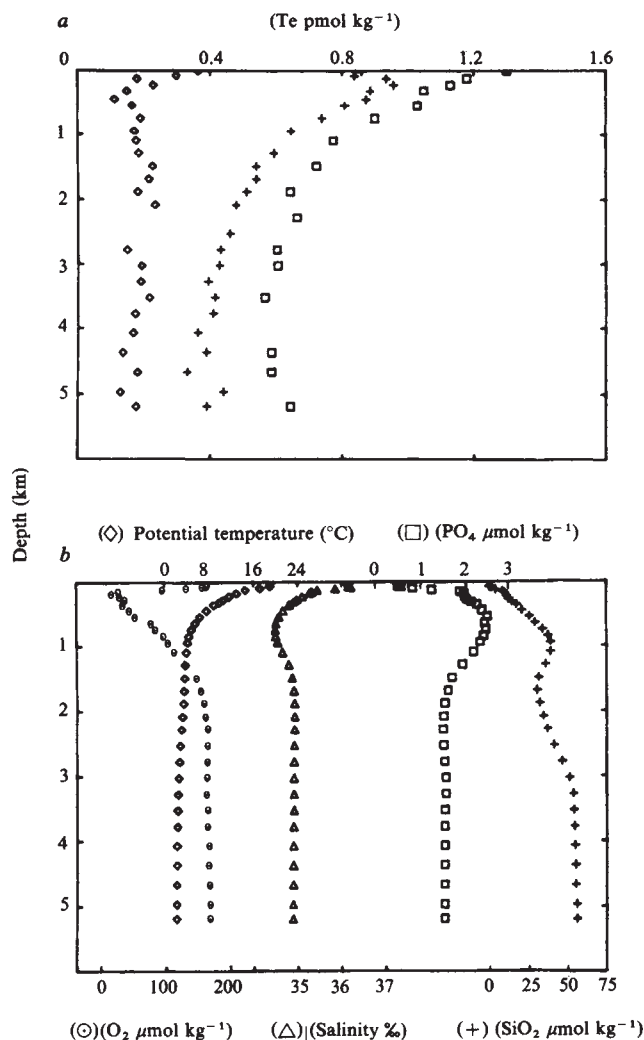


Fig. 1 *a*, Profiles of Te(IV)($\diamond$), Te(VI)(+) and total Te($\square$) and *b*, associated hydrographic data from Long Lines 23 ($14^{\circ}59' \text{S}$, $01^{\circ}00' \text{E}$) in the Angola Basin of the eastern South Atlantic. The analytical precisions for Te(IV), Te(VI) and total Te were 5.6, 8.7 and 4.8%, respectively; the limit of detection was 0.02 pM for all species based on a 1-l sample volume. Standard addition experiments showed quantitative recoveries for Te(IV) and Te(VI).

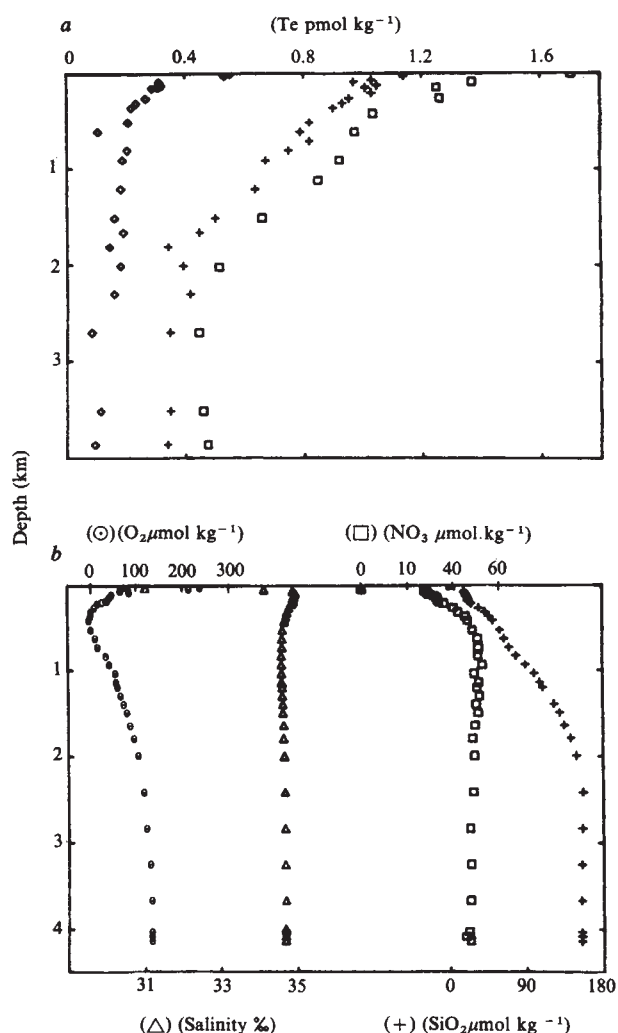


Fig. 2 *a*, Profiles of Te(IV)($\diamond$), Te(VI)(+) and total Te($\square$) and *b*, hydrographic parameters from the Panama Basin. Note the very low surface salinity and the very intense oxygen minimum. ($07^{\circ}00' \text{N}$, $78^{\circ}40' \text{W}$).

levels are constant. Here Te(VI) has a mixed layer concentration of 0.86 pM and shows a slight maximum in the shallow oxygen minimum, which is probably of advective origin from the region of intense upwelling off Namibia. Below 400 m Te(VI) parallels closely the total Te distribution dropping to Deep and Bottom Water values of 0.43 pM. The thermodynamically stable reduced form, Te(IV), has a surface concentration of 0.36 pM, the levels dropping slightly into the oxygen minimum (0.12 pM) and rising below this, being uniform (0.2 pM) between 1,500 and 3,500 m. There is some indication of a drop in the concentrations in the Antarctic Bottom Water that fills the deeper part of the Basin and concentrations in the lower part of the water column scatter about a value of ~ 0.15 pM. LL23 was originally chosen as a site for analysis because the chemical signatures of the various water masses of Antarctic and North Atlantic origin are strongly expressed there (Fig. 1*b*). However, except in the shallow oxygen minimum, these signatures are not evident in the distribution of Te and its oxidation states. Yet the extrema in Te(VI) and Te(IV) are in the opposite sense from what would be expected on purely thermodynamic grounds. The profile is dominated overall by the input of Te, predominantly as Te(VI), to the surface layer and its removal from the water column by scavenging. These two processes must have time constants much shorter

than those of the regional physical circulation, otherwise concentration anomalies of purely advective origin (as for the major nutrients; Fig. 1*b*) would be observed. The fact that the Te(VI) profile drops smoothly across the core of the Antarctic Intermediate Water indicates that the particle interactions are to some degree reversible, as is the case for Po.

The Panama Basin is an area of very high biological productivity as evidenced by the extreme oxygen depletion of the mid-thermocline waters (Fig. 2*b*). The mixed layer has a very low salinity caused by the large local excess of precipitation and continental run-off over evaporation. Below 1,000 m, the water column is occupied by the North Pacific Deep Water and the effective sill depth of the basin is $\sim 2,500$ m. In form the Te profiles reported here are very similar to those from the eastern South Atlantic (Fig. 2*a*); surface concentrations are slightly higher for both species (1.14 and 0.55 pM), perhaps indicating an input of Te with the fresh water. The intense oxygen minimum finds no obvious expression, however, unlike at LL23. Te(IV) concentrations are constant at ~ 0.2 pM between 500 and 2,500 m and drop to 0.10 pM in the ponded bottom waters; Te(VI) levels drop smoothly from the surface to 0.35 pM at 2,000 m and are quite constant below this. Unlike most other measured chemical species^{1,2}, these Deep Water concentrations are lower than those

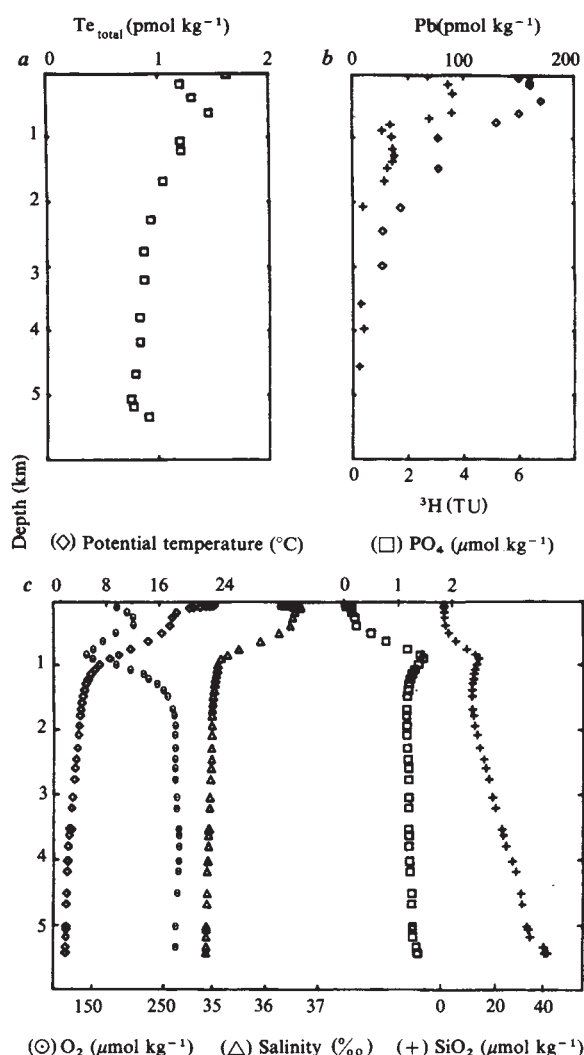


Fig. 3 *a*, Total Te and *c*, hydrographic data at TTO 241 (36°17' N; 57°23' W) and *b*, profiles of Pb (◇) and ^3H (+) in the western Sargasso Sea. The Pb profile is located at 34°15' N, 66°17' W. The ^3H profile is from TTO 6 at 34°39' N, 67°21' W.

in the South Atlantic by ~40%, which is strong evidence for rapid removal of Te by scavenging from the water column.

To confirm this interpretation, the total Te distribution has been determined at TTO 241 in the western North Atlantic (Te(IV) and Te(VI) were not measured because of the limited sample volume; Fig. 3). The concentrations are substantially higher here at all depths than at LL 23, except in the uppermost thermocline. As TTO 241 is relatively close to the formation areas of the water masses that make up the North Atlantic Deep Water and as these are relatively 'young' there, the elevated levels of Te in the subthermocline waters are probably of advective origin. Assuming that the surface waters of the North Atlantic are enriched generally in Te then, as the source waters for the convectively produced North Atlantic Deep Water, they will carry this imprint into the deep interior. As the water masses propagate away from their formation areas, Te is removed progressively by scavenging processes leading to the decrease in its Deep Water concentration observed between TTO 241 and LL 23, similar to the behaviour shown by Al in this region and, to a lesser extent, by Be⁷. The high concentrations in the lower few hundred metres are probably caused by the dissolution of particles from the well developed nepheloid layer in the unfiltered acidified water samples, an identical feature to that observed for Be in these samples¹⁰.

There are several similarities between the Te profile at TTO 241 and those reported for Pb and ^3H at adjacent stations

in the Sargasso Sea (ref. 11 and W. Jenkins, personal communication) (Fig. 3). The concentration of Pb in the surface waters shows seasonal variations in this region (E. A. Boyle, personal communication). In the summer months the aeolian input accumulates to elevated concentrations in the shallow mixed layer over the seasonal thermocline, but mixing by winter storms obliterates this feature, dissipating the enrichment into the waters above the main thermocline. A similar effect can be seen for Te (whose concentration at 150 m (1.20 pM) is substantially lower than at the surface (1.60 pM)). Pb, ^3H and Te all show sub-surface maxima in the depth range 400–600 m, caused in the case of ^3H , by the combination of the decrease in the input flux with time (that is, the older waters have higher levels) and advective processes. For Pb and Te, it may be caused by advection from northern areas with higher input fluxes or by shallow regeneration of material scavenged in the surface layers. The extrema for Pb and Te are not coincident, which does not appear to be an artefact of the sampling density or of the lateral separation of the stations. The Pb maximum lies in the 18°C water; that for Te at ~14°C, immediately above the sharp maximum in the nutrients, an advective feature associated with the distal end of the northward propagating Antarctic Intermediate Water. Although shallow regeneration of ^{210}Pb is observed in the oceanic water column⁸, the Pb maximum could be an advective feature as the 18°C water is produced in winter off the north-east coast of the United States close to the dominant source of anthropogenic Pb. Either the Te source function is somewhat different from that for Pb, leading to greater relative enrichments in more northern latitudes and hence a deeper advective maximum, or this feature is indeed caused by shallow regeneration processes. More data will be required to distinguish between these possibilities.

Throughout the western North Atlantic the ^3H profiles show a sharp minimum at about 900 m, the core of the Antarctic Intermediate Water, and a secondary maximum beneath it. This feature lies above the Mediterranean and Labrador Sea Waters and appears to be derived from the northern gyre. Although the existing sampling density for Pb and Te does not resolve the Antarctic Intermediate Water core (Fig. 3), both elements show a break in their vertical gradients in the ^3H maximum, probably indicating the presence of an advective component. Below 1,500 m the profiles of all three species decrease steadily to uniform values below 2,500 m. Based on numerous profiles collected world-wide, the residence time of ^{210}Pb can be estimated as <1 yr in the mixed layer and ~50 yr in the deep waters⁸. As the vertical gradient in the 'age' of the various water masses is greater than this, advective features can only be maintained against removal of the element in the more rapidly circulating upper waters. In the interior, scavenging is completely dominant.

The sources of the Te flux to the ocean surface, aeolian and/or fluvial, are not yet known and given the complete lack of information on the environmental chemistry of Te it is not possible to estimate what proportion, if any, of the flux is anthropogenic.

Te and Se both occur throughout the oceans in two oxidation states; the major component of each is the oxidized form, but whereas for Se this is the thermodynamically stable species, the reverse is true for Te. The vertical distributions of the two elements are very different in shape, with Se displaying a nutrient-type distribution resulting from biological uptake at the surface and regeneration at depth, and Te showing surface enrichment and deep depletion resulting from input at the surface and scavenging at depth. The deep water distribution of Te is controlled primarily by scavenging, as opposed to water mass advection, owing to a residence time much shorter than the ocean turnover time, whereas that of Se reflects the advective processes. The high geochemical reactivity of Te resembles more closely ^{210}Po than Se; the reported residence time of ^{210}Po , the heavier homologue of Te, is 0.6 yr at the mixed layer⁸. Although Se(VI) and Te(VI) have the same oxidation state, they have different chemical structures; selenate, like sulphate, has tetrahedral coordination, SeO_4^{2-} , whereas tellurate is octahedral,

Te(OH)₆. Thus, from the structural point of view, tellurate should behave like Sn or Po, which exist in sea water as Sn(OH)₄ and Po(OH)₄ (ref. 12) and are geochemically very reactive, rather than like Se.

As the penetration of the anthropogenic transient tracers (³H, ¹⁴C, freons, etc.) into the interior of the North Atlantic continues to be monitored, this ocean basin will be developed into a time-calibrated laboratory for the study of the behaviour of elements with short oceanic residence times. It should be possible to quantify the mechanisms by which reactive elements, such as Al, Be, Bi and Te, are removed. As these elements are analogues of the reactive pollutants being introduced to the ocean, quantitative understanding of their behaviour will be useful for predicting the effects of the pollution.

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Late Quaternary hydrological changes in the Gulf of Carpentaria

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The Gulf of Carpentaria is a shallow epicontinental sea between Australia and Papua New Guinea (Fig. 1); it has a maximum depth of -69 m and is connected to the Arafura Sea across a -53-m sill¹; in the east, the connection to the Coral Sea through Torres Straits is -12 m (ref. 1). We propose here that low sea levels during the Pleistocene^{2,3} (Fig. 2) resulted in subaerial exposure of the Gulf of Carpentaria, thereby reducing the regional input of moisture and latent heat to the atmosphere and tropical cyclogenesis. As a result, the climate of the Carpentaria Basin may have been significantly drier than at present^{4,5}. Even so, previous workers^{6,7} have reported the presence of lacustrine sediments in the Gulf that could have been deposited when the sea level dropped below the -53-m contour¹. During 1982, the extent of Lake Carpentaria was defined by high-resolution seismic profiles and cores. The size and development of Lake Carpentaria and its dependence on the hydrological balance are discussed below.

We conducted two cruises in the Gulf of Carpentaria during 1982 to investigate the former presence of Lake Carpentaria, determine its salinity and size, and to establish its correlation with the glacial sea-level record. More than 1,600 km of high-

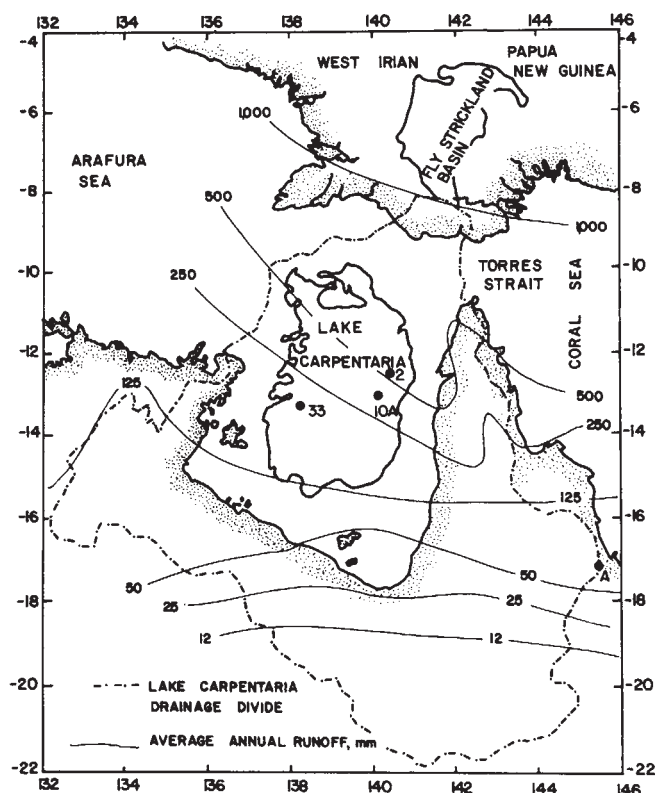


Fig. 1 The Gulf of Carpentaria region showing the maximum extent (-53-m shoreline) of Lake Carpentaria during the last glacial maximum and the extent of the Lake Carpentaria drainage basin. Full bathymetry is given elsewhere¹. Core locations for GC2, GC10A and GC33 are shown. The Atherton Tableland data¹⁵ are from point 'A'. Overlain on the geographical map are the contours of present average annual runoff as deduced from the literature^{12,13}. Land-based runoff data have been interpolated across the Gulf. At present, half of the runoff comes from the area between the 500 and the 1,000-mm isoline. Straightening the 500-mm isoline causes a 10% decrease in the integrated runoff.

resolution seismic profile was obtained, including five east-west seismic profiles in the region 12°-14° S and 139°-141° E, a complete east-west transect of the Gulf at ~13° S and a complete north-south transect of the Arafura Sill¹. These profiles show the common presence of a (nominally) -63-m and a -53-m shoreline morphological feature under <1 m of marine sediment, confirming our preliminary work¹. (Hydroisostatic adjustment and bathymetric errors imply that all depths must be nominal.) Across the Arafura Sill, the youngest erosion surface occurs beneath <3 m of sediment, but earlier erosional surfaces are comparatively deeply incised.

Of the 35 cores obtained with good seismic control, Fig. 3 shows the lithology of three key cores. Locations are given in Fig. 1. Environmental interpretations are based on facies, lithology, faunal composition, carbon-14 dates from Unit IV and the sea-level curves of Fig. 2.

Unit I is a grey-green soft shelly and sandy mud containing marine molluscs, bryozoans, marine ostracods (*Cyprideis*, *Polycope*, *Bairdia*) and marine foraminifera (*Pseudorotalia inflata*, *Heterolepa subhaidingeri*, *Neoponides schreibersi*, *Ammonia beccarii*, *A. convexa*, *Quinqueloculina philippinensis*, *Textularia agglutinans*) and was deposited in the marine environment of the modern central gulf.

Unit II is a dark grey firm mud containing ostracods (principally *Ilyocypris* and *Cyprideis*) and foraminifera indicative of brackish (*A. beccarii*, *A. beccarii tepida*, *A. convexa*) to saline water (*A. beccarii*, *A. convexa*, *P. inflata*, *H. subhaidingeri*, *Elphidium hispidulum*). Recalcified ostracods indicative of subaerial exposure were identified in Unit II of GC33 collected at

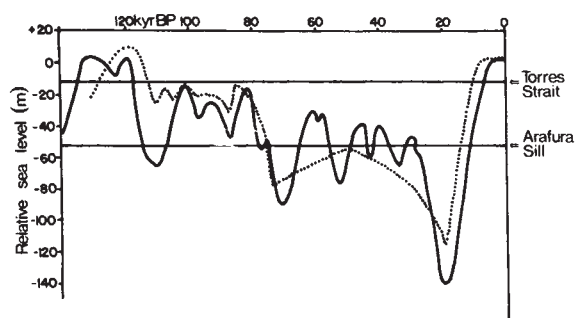


Fig. 2 The sea-level curves applicable to the Gulf of Carpentaria with the sill heights of Torres Strait and the Arafura Sill¹ indicated. The solid curve is from the Huon terraces² and the dotted curves are from the stable-isotope record of benthic foraminifera³. Note that hydroisostatic adjustment of the Carpentaria Basin will have a small effect on the sill heights as will errors in the bathymetric data¹.

a present water depth of -58 m. In cores GC2 and GC10A from -67 m, no evidence was found of subaerial exposure of Unit II. Unit II appears to have been deposited in a lake whose surface level varied between the -58 and -67-m contours and whose salinity varied from brackish to saline.

Unit III is a finely laminated sediment containing calcite crystals of the order of 20 μm or less probably representing authigenic calcite precipitation. *A. beccarii tepida* are predominant and environmental stress is indicated. *Cyprideis* show definite marks of bleaching, dissolution and iron sulphides implying anoxic deposition. Unit III is probably analogous to the 'seekreide' of the Black Sea⁸ and the laminae of Green Lake⁹ and Lake Zurich¹⁰.

Unit IV is a shell hash that has been radiocarbon dated at 26.1 ± 0.6 kyr (95-106 cm) to 36.14 ± 2.9 kyr (115-126 cm). It is composed mainly of *Americanna*, *Gyraululus* and *Corbiculina* shells and the foraminiferal assemblage comprises abundant *A. beccarii* and *A. convexa* and rare, possibly reworked *P. inflata*. *Cyprideis* is the predominant ostracod. The fauna indicate brackish water conditions.

Unit V is a slightly mottled dark-grey fine-grained mud with marine to brackish water ostracods (principally *Cyprideis* with *Cytherella*, *Monoceratina*, *Uroleberis*) and a marine foraminiferal assemblage (*P. inflata*, *A. beccarii*, *A. convexa*). This unit was probably deposited in a highly brackish environment but has undergone subaerial weathering. Soil formation occurs within the unit.

These results support the existence of Lake Carpentaria during the low sea level of the last glacial maximum and a hydrological balance for the lake can, therefore, provide a measure of tropical continental climates.

The present climate of the Lake Carpentaria drainage basin (as defined in Fig. 1) is dominated by rainfall between December and March. Mean annual rainfall is highest in southern Papua New Guinea ($2,500 \text{ mm yr}^{-1}$)¹¹ where rainfall arrives on the north-west monsoon but decreases rapidly with increasing latitude to $\sim 500 \text{ mm yr}^{-1}$ in the southwestern portion of the basin¹² where rain is brought by the south-east trade winds. Sporadic tropical cyclones also cause considerable rainfall. Figure 1 shows the present average annual runoff yield^{12,13} in the Lake Carpentaria Basin and the runoff input (R) to the Lake Carpentaria drainage basin based on these conditions is $2.3 \times 10^{11} \text{ m}^3 \text{ yr}^{-1}$. Evaporation (E) for the present Gulf of Carpentaria is estimated as $1,653 \text{ mm yr}^{-1}$ (ref. 14).

A simple hydrological balance for a steady-state Lake Carpentaria can be expressed as

$$R - E(A_L) = 0 \quad (1)$$

where A_L is the area of the lake at depth contour L , the correction for precipitation directly to the lake surface being small. For a lake surface at -53 m which overflows, the residence time (t)

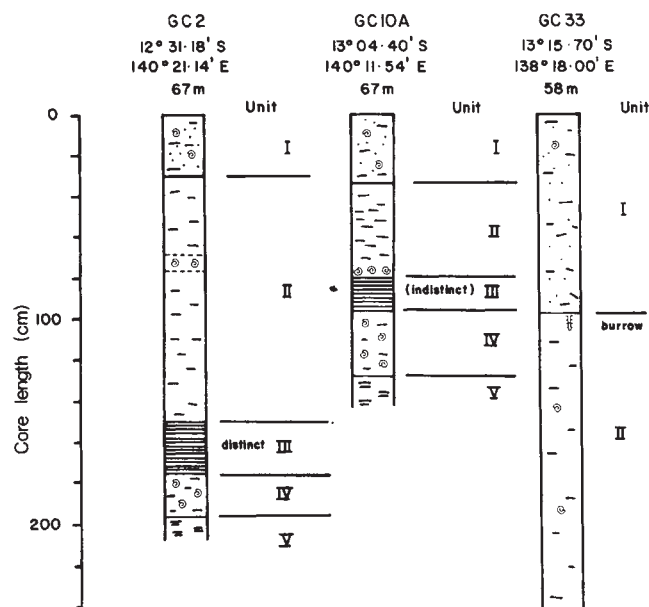


Fig. 3 Core logs, locations, and present water depths for cores used in this study. Radiocarbon dates on Unit IV of GC10A range from 36.140 ± 2.9 kyr (ANU 3692, 115-126 cm) to 34.7 ± 1.6 kyr (ANU 3692, 106-115 cm) to 26.100 ± 0.56 kyr (ANU-3691, 95-106 cm). Although Unit III occurs below Unit II in these cores, other cores (GC1, GC3) show Unit III in Unit II, albeit very near the base of Unit II. Preliminary interpretation of this occurrence is a variation in the depth of the anoxic bottom water. See text for a full description of the units and their environments.

of runoff in the overflowing lake is

$$V_{53}/(R - E(A_{53})) = t \quad (2)$$

where A_{53} and V_{53} refer to the area and volume of a Lake Carpentaria with a surface at the present -53-m contour¹. Figure 4 shows both the R versus E conditions for a Lake Carpentaria of various surface areas¹ and the R versus E conditions for an overflowing lake at the -53-m level labelled with respect to the residence time of freshwater in the lake according to equation (2).

The differing states of preservation of ostracods in GC2 and GC33 show that the lake which deposited Unit II varied in depth and did not always extend to the present -58-m contour. The deposition of Unit II in Lake Carpentaria therefore occurred with an R/E value of $<60\%$ of present (Fig. 4) in general agreement with other estimates^{4,5,15-17}, but Lake Carpentaria was brackish, which required the salt of trapped seawater to be removed. (35% seawater, trapped as sea level fell below -53 m, would precipitate halite on evaporation to -61 m (ref. 1).)

If one accepts the $\delta^{18}\text{O}$ sea-level curve³ (Fig. 2), then the Arafura Sill was above sea level and the Carpentaria Basin was isolated from ~ 75 to ~ 10 kyr BP. The Fly River of Papua New Guinea flowed formerly into the Lake Carpentaria Basin¹⁸; this flow could have cut the deep buried channels seen in the seismic profiles across the Arafura Sill. The salt of trapped seawater could, therefore, have been removed by continuous flow to the sea. Subsequent diversion of the Fly River by the Oriomo uplift¹⁸ (before 36 kyr) and infilling of the sill channels would then result in the presently observed morphology.

If the sea-level record of Bloom *et al.*² is accepted (Fig. 2), then increased runoff would be required to remove the salt from the -50-m sea-level high at 30 kyr. If a residence time (mean flushing time) of 10 yr is accepted as reasonable, then R/E must have increased by 1.5 times its present value (Fig. 4) and the much diminished Gulf of Carpentaria (A_{50}) could have existed as an estuarine embayment laying down 'lake-like' sediments and accounting for the lack of an obvious marine sequence. The Atherton data of Kershaw¹⁵ indicate that south-east trade

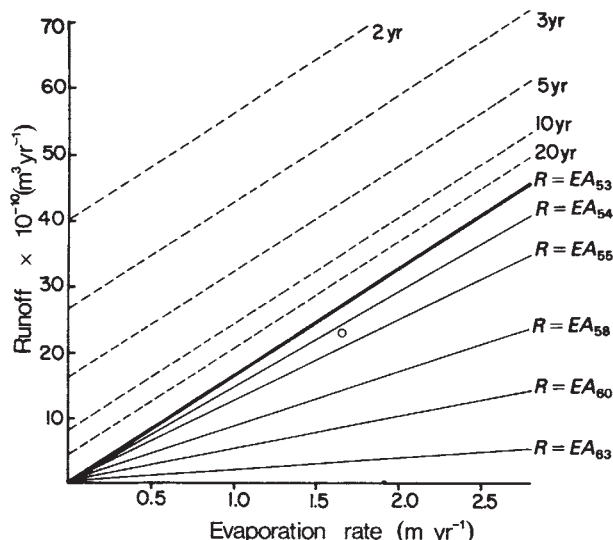


Fig. 4 A simple hydrological balance for a steady-state Lake Carpentaria based on equation (1) is shown with the solid lines. O, Present total runoff (R) from Fig. 1 and present evaporation (E)⁹, (10% error in R and E would be a good estimate.) Given present runoff and evaporation conditions, an exposed closed-basin Lake Carpentaria (by sea-level fall) could maintain a lake with a surface area between the present -55 and -54-m contours. Evaluation of GC33 indicates a late Quaternary Lake Carpentaria oscillating about the -58-m contour (A_{58}). Also shown are the residence times for freshwater in an estuarine embayment overflowing the -53-m contour (dotted lines). Such an overflow may be necessary to flush the salt of seawater from the basin to prevent hypersaline conditions. At present evaporation rates and assuming a 10-yr residence time of freshwater, a 50% increase in runoff may be appropriate to flush sea salt from the lake basin at times of falling sea level.

winds rainfall was much reduced at this time. Therefore, if increased R/E ratios correspond to the sea-level high at ~30 kyr (ref. 2), they are probably best explained by increased effectiveness of the north-west monsoon.

We therefore suggest the following scenario for the late Quaternary of the Gulf of Carpentaria: before 36 kyr BP, the Carpentaria Basin contained a marine to brackish water body (Unit V), followed by a period of minimum rainfall/runoff and subaerial exposure which allowed the formation of a soil in Unit V. From 36 to 26 kyr BP, a fresh to brackish water body is indicated by the fauna of Unit IV; for a short but undetermined period after 26 kyr BP, the basin contained a fresh to brackish water body subject to periodic authigenic calcite precipitation (Unit III). These 'whittings' were preserved as laminae by the anoxic sediments of a stratified and morphologically controlled water body. A seismic 'shoreline' at -63 m may be indicative of the extent of the anoxic bottom layer. From 26 to ~10 kyr BP, the basin contained a brackish to saline Lake Carpentaria with a variable water level with R/E probably about 60% of the present. After ~10 kyr BP, rising sea level crested the Arafura Sill and gradually restored the basin to fully marine conditions.

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Two contrary modes of chemolithotrophy in the same archaeobacterium

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Sulphur-dependent archaeobacteria, which are found around nearly boiling continental solfataric springs and mud holes, can be assigned to two distinct branches: the aerobic, sulphur-oxidizing *Sulfolobales*¹⁻⁴ and the strictly anaerobic sulphur-reducing *Thermoproteales*⁵⁻⁷. Here, we report the isolation of a group of extremely thermophilic solfataric archaeobacteria that are able to grow either strictly anaerobically by reduction, or fully aerobically by oxidation of molecular sulphur, depending on the oxygen supply. We have also established that the ability to grow in these two ways is shared by *Sulfolobus brierleyi*, a well-known less thermophilic sulphur-oxidizing archaeobacterium capable of ore-leaching⁸. The phenomenon may be dependent on a fundamental switch in genome expression. These organisms might represent the primitive forerunners of sulphur-oxidizing archaeobacteria, meeting their energy requirements either by oxidation or by reduction of the same element.

To look for previously unknown thermophiles, we collected aerobic and anaerobic⁶ samples from mud holes with pH values between 1.5 and 4.5 and temperatures between 85 and 93 °C, located in the Solfatara and Pisciarelli Solfatara craters, both in Pozzuoli, Italy, where *Sulfolobus solfataricus* was first isolated^{3,9}. The samples were brought to the laboratory without their temperature being controlled. Anaerobic mineral medium^{10,11}, pH 3, was supplemented with sulphur (0.1%) and yeast extract (0.02%) and was inoculated with the samples (5%

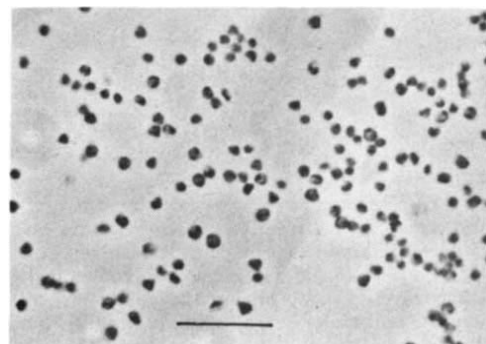


Fig. 1 A phase-contrast micrograph of cells of Solfatara isolate So 4a, grown anaerobically (= So 4a/H₂). Scale bar, 10 µm.

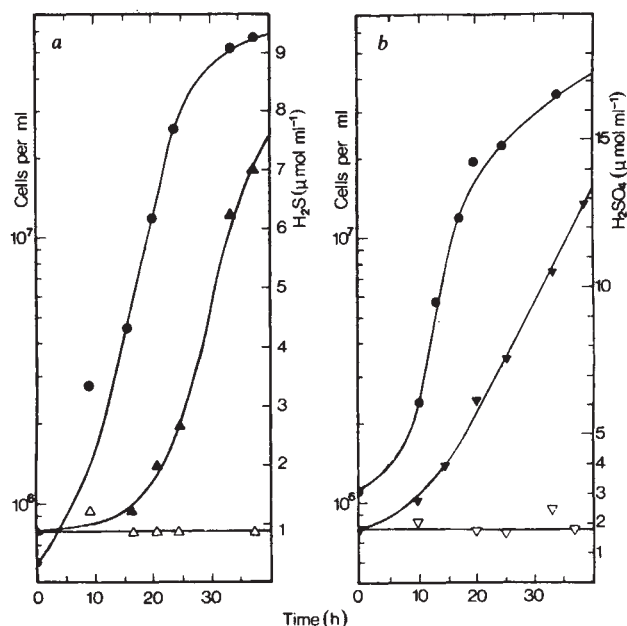


Fig. 2 Anaerobic (a) and aerobic (b) growth of isolate So 4a. Panel a shows: ●, cell growth, as determined microscopically using a Thoma counting chamber; ▲, H₂S formation during growth as determined by titration after precipitation as cadmium sulphide¹⁶; △, H₂S in the uninoculated medium. Panel b shows: ●, cell growth; ▼, H₂SO₄ formation; ▽, H₂SO₄ in the uninoculated medium.

Methods. a, Cells were grown anaerobically¹⁰ in 20-ml cultures in 100-ml stoppered serum bottles in the mineral medium of Allen¹¹ with the addition of 0.2 g l⁻¹ yeast extract (not essential, but stimulatory (H₂SO₄) to growth) and 1 g l⁻¹ elemental sulphur. The pH was adjusted to 2.5 and remained constant during growth. The culture was shaken (150 r.p.m.) at 82 °C with H₂/CO₂ (80:20; 3 bar) as gas phase. Reduction of the medium was effected with Na₂S¹⁰. b, Aerobically grown cells in the same medium as a, but not reduced by sulphide. The culture was shaken at 150 r.p.m. at 82 °C in tall cylindrical glass vessels in the presence of air. The pH, initially adjusted (H₂SO₄) to 3.5, dropped during incubation due to H₂SO₄ formation. H₂SO₄ was determined by titration with 0.1 M NaOH (phenolphthalein as indicator). For a positive identification, H₂SO₄ was precipitated before and after the experiment as barium sulphate and determined gravimetrically.

inoculation). After incubation for 3 days at 85 °C in an H₂/CO₂ atmosphere (80:20 v/v, 3 bar pressure) and with strict exclusion of O₂, irregular spherical organisms (Fig. 1) grew to a density of about 10⁷ ml⁻¹. No growth occurred in the presence of H₂ and S at a redox potential around 0 mV, as determined by the indicator resazurin. After a few transfers in the same medium, the final cell yield was improved to about 3 × 10⁸ ml⁻¹, possibly due to adaptation to the medium. Growth was strictly hydrogen- and sulphur-dependent, similar to *Pyrodicticum*⁶ and *Thermoproteus neutrophilus*⁷. Yeast extract was not essential, but increased the cell yield. During growth (Fig. 2a), large amounts of H₂S were formed (up to 15 μmol ml⁻¹), indicating that the cells were thriving due to their hydrogen/sulphur autotrophy⁷. The cultures were purified by four successive serial dilutions. Growth occurred within a temperature range between 65 and 95 °C with an optimum around 90 °C and in a pH range of 1 to 5 with an optimum ~2. Surprisingly, four of the new isolates (So 4a, PS 2c, PS 7c, PS 11c) were able to grow in the absence of hydrogen, but when shaken in the presence of air in open culture vessels. Under these aerobic conditions, the pH of the culture was greatly lowered during growth due to the formation of sulphuric acid (Fig. 2b). Therefore, the new isolates, growing aerobically, showed the same type of autotrophic sulphur oxida-

tion as carried out by *Sulfolobus*¹. In contrast to known *Sulfolobus* species³, the novel isolates do not grow on organic material in the absence of sulphur. One isolate (So 4c/k), however, formed only low amounts of sulphuric acid aerobically but could not be transferred successfully under aerobic conditions. Further, we recently isolated similar-looking organisms, which grew anaerobically by hydrogen-sulphur autotrophy, from the acidic springs of Furnas Caldeiras and Ribeira Grande on the Azores. These organisms, however, were unable to grow aerobically or to form sulphuric acid.

To establish whether the cultures were pure, the aerobically grown isolates from Italy were serially diluted four times and incubated in the presence of oxygen. All subcultures obtained after this procedure were still able to grow aerobically and anaerobically on sulphur. Moreover, parallel serial dilutions and subsequent incubations of the same anaerobic culture, performed under aerobic and anaerobic conditions, gave rise to the same cell titre of about 10⁸ ml⁻¹, indicating that practically all the cells in the original culture must have been able to grow under both sets of conditions. However, anaerobically and aerobically grown cells of So 4a, which was the first isolate to be obtained in a pure state, showed significant differences: the diameter of the aerobes was about 1.5 times greater than of the

Table 1 DNA homology between the new isolates and *Sulfolobus brierleyi* and *Sulfolobus acidocaldarius*

	Filter-bound DNA from:				
	So 4a/O ₂	So 4a/H ₂	So 4c/k	<i>S. brierleyi</i>	<i>S. acidocaldarius</i>
³² P-labelled DNA from:					
So 4a/O ₂	(100)	94	84	9	12
So 4a/H ₂	100	(100)	89	13	14
So 4c/k	81	88	(100)	7	7
<i>S. brierleyi</i>	24	9	ND	(100)	9
<i>S. acidocaldarius</i>	23	9	ND	13	(100)

DNA homologies among the Solfatar (So 4) isolates, *Sulfolobus brierleyi* (DSM 1651), and *Sulfolobus acidocaldarius* (DSM 639) were assessed in DNA/DNA hybridization experiments. So 4c/k and So 4a/H₂ were anaerobically grown isolates, So 4a/O₂ was the aerobically grown line of isolate So 4a/H₂. Filters (6 mm diameter), each containing about 10 μg single-stranded DNA¹⁸, were prepared after alkaline denaturation of the DNA^{19,20}. ³²P-labelled DNA was prepared *in vitro* by nick translation²¹ with ³²P-deoxyadenosine triphosphate and a reagent kit (8160 SB, Bethesda) after shearing by sonication (3 × 2 s; 30 W; Branson). Radioactivity was determined in a scintillation spectrophotometer (Betascint BF 5000, Berthold). The specific activities obtained for the different DNAs were (c.p.m. per μg × 10⁻³): So 4c/k, 111; So 4a/O₂, 92; So 4a/H₂, 158; *Sulfolobus brierleyi*, 179; *Sulfolobus acidocaldarius*, 207. For hybridization, 0.27 μg of heat-denatured, radioactively labelled DNA was incubated with the DNA-containing filters in a 3 × SSC buffer for 24 h at 65 °C. The amount of c.p.m. bound in the homologous filters was set as 100% homology (for example, 6,000 c.p.m. for So 4a/O₂) and compared with the activity of the heterologous filters. ND, Not determined.

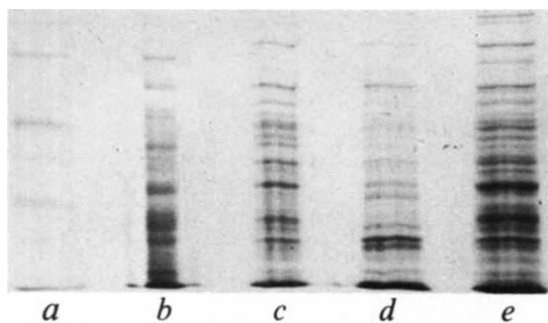


Fig. 3 Protein patterns of crude extracts, obtained after electrophoresis in a SDS-polyacrylamide gel. Supernatants of the centrifuged (1 h; 150,000 g) crude extracts were applied onto a polyacrylamide gel (7.5% w/v)¹⁷. After electrophoresis, protein was stained with Coomassie blue. *a*, *M*_r standards: β -galactosidase (135,000); phosphorylase *a* (92,500); bovine serum albumin (68,000); ovalbumin (45,000). *b*, *S. acidocaldarius* (30 μ g of protein). *c*, Isolate So 4c/k (which grows only anaerobically; 18 μ g of protein). *d*, Isolate So 4a/O₂ (aerobically grown; 12 μ g of protein). *e*, Isolate So 4a/H₂ (anaerobically grown; 12 μ g of protein).

anaerobes; packed cells of the aerobes had an ochre colour, whilst the anaerobes were greenish-black; and the protein patterns of crude extracts of cells grown aerobically and anaerobically differed significantly (Fig. 3*d, e*).

To determine whether the cells grown aerobically and anaerobically were genetically identical, we prepared DNA¹² from both types of cells, which had been cultured in bulk in a 100-litre, enamel-protected fermenter (HTE, Bioengineering, Wald, Switzerland). DNA preparations from both had the identical G+C-content of 31 mol%, as determined by the *T_m* method¹³. Further, the two DNAs, when mixed, yielded a single sharp melting curve (data not shown). DNA hybridization of the two types of So 4a (Table 1) confirmed their genetic identity, as DNA from aerobically grown cells (So 4a/O₂) showed 100% homology with that from the anaerobically grown cells (So 4a/H₂). In the reciprocal hybridization experiment, 94% homology was shown, and this, in the range of accuracy of this method, also indicates the identity of both genomes¹⁴. Another isolate, So 4c/k, which was unable to grow aerobically, showed a high degree of DNA homology with both aerobically and anaerobically grown cells of So 4a (Table 1), indicating membership of the same species¹⁴.

A protein with an apparent relative molecular mass (*M_r*) of 82,000 was among the soluble proteins obtained after centrifugation of crude extracts of both aerobically and anaerobically grown forms of So 4a. This protein could be ADP-ribosylated by diphtheria toxin, suggesting that So 4a is an archaeobacterium and the modified protein elongation factor 2, this reaction being characteristic of archaeobacteria¹⁵. In the present case, the reaction was influenced by the culture conditions: supernatant protein from anaerobically grown cells was ADP-ribosylated at a threefold higher rate than was an equal amount of protein from cells grown in the presence of oxygen.

The new isolates appeared to be related to the sulphur-dependent archaeobacteria by virtue of their extremely high temperature optimum, their metabolism and their shape. Screening of members of this group revealed that *Sulfolobus brierleyi* like our new isolates, can also grow by anaerobic hydrogen-sulphur autotrophy, a mechanism hitherto unknown in this organism. Although the G+C content of its DNA is identical to that of our isolates³, *S. brierleyi* has a temperature optimum 20 °C lower than that of the new isolates. DNA/DNA hybridization with isolate So 4a revealed a degree of homology (Table 1) well below the limits of significance¹³, indicating that the two organisms belong to different taxa above the species level, that is, distinct genera or families. In contrast to *S. brierleyi*, *S.*

acidocaldarius and *S. solfataricus* were unable to grow anaerobically. Moreover, *S. brierleyi* differed from the other two species in its G+C-content and, in a DNA-DNA hybridization performed with one of them (*S. acidocaldarius*; Table 1), no homology was found. Anaerobic sulphur-reducing archaeobacteria, including *Thermoproteus tenax*, *T. neutrophilus*, *Desulfurococcus saccharovorans*, *Thermofilum* sp. V 24N, *Pyrodicticum occultum* and *Thermophilus maritimus*, were not able to grow aerobically by sulphur-oxidation. Furthermore, these organisms have a G+C content some 20 to 30% higher than *S. brierleyi* and the new isolates (refs 5, 6 and K.O.S. and F. Fischer, unpublished data).

This is the first evidence that a highly anaerobic organism that requires the same low redox potentials as methanogens, can also grow fully aerobically after changing its metabolism into another type of chemolithotrophy, exploiting an energy yielding pathway with an entirely different direction. Many proteins seem to be involved in this metabolic switch. The inability of the new isolates to grow using hydrogen at redox potentials of around 0 mV may reflect an extreme oxygen sensitivity of the anaerobic 'set' of enzymes. The occurrence of this switch in metabolism will allow the study of induction phenomena in thermophilic archaeobacteria.

Since submitting this manuscript, we have learned that W. Zillig is working independently on a *Sulfolobus*-like isolate which is also able to grow anaerobically on H₂ and S.

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Plasmid-related anaerobic autotrophy of the novel archaeobacterium *Sulfolobus ambivalens*

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Three different species of the genus *Sulfolobus*¹, *S. acidocaldarius*, *S. solfataricus* (= *Caldariella*)² and *S. brierleyi*³, have been distinguished⁴ by the conditions required for optimal growth, by the component patterns of their DNA-dependent RNA polymerases and by DNA sequence data. Many isolates of these species are able to grow chemolithoautotrophically using CO₂ as the sole

carbon source and the oxidation of S(0) with O₂ yielding sulphuric acid, as the energy source, though a few others grow only heterotrophically. We show here that a strain of a novel *Sulfolobus* species, *S. ambivalens*, is alternatively able to live by an anaerobic mode of chemolithoautotrophy, also using CO₂ as the sole carbon source, but using reduction of S(0) with H₂, yielding H₂S as the energy source. This mode of growth is correlated with the amplification of a plasmid, pSL10.

The obligatorily autotrophic isolate L10 of *S. ambivalens*, found in a solfataric waterhole at the Leirhnukur fissure, Iceland, (94 °C, pH 2.3) first appeared, besides *Thermoproteus*, in enrichment cultures in which elemental sulphur and hydrogen were offered as the energy source and CO₂ as the sole carbon source. The strain was cloned by serial dilution and from colonies grown on 12% starch plates in a soft layer containing 2% colloidal sulphur in 9% starch in Allen's medium⁵ with 80% H₂ and 20% CO₂ in the gas phase.

The strain grows in Allen's medium⁵, on S(0), with air to which 5% CO₂ was added as sole carbon source, producing H₂SO₄, with a generation time as low as 4 h at pH 2.5. Alternatively, it multiplies in the absence of oxygen on S(0), but with H₂ and CO₂, producing H₂S with a generation time of ~8 h at pH 3.5, and, in the presence of 0.005% yeast extract, of 4 h.

Neither the presence of oxygen at the outset, nor subsequent aeration of the culture, influence reductive growth. But, in reverse, oxidative growth of a culture, previously kept reductively, starts only after a phase lag of several hours.

Several other isolates from Icelandic solfataras are also able to grow via sulphur reduction. Segerer, Stetter and Klink have independently made similar observations with type strain DSM 1651 of *S. brierleyi* and with different isolates of undetermined phylogenetic status⁶.

L10 cells grown with H₂ yield the same DNA *Bam*HI-restriction fragment pattern as L10 cells grown with O₂, except that two fragments appear strongly amplified (Fig. 1, lane 4). The DNA-dependent RNA polymerases, isolated as described previously^{4,7}, yield identical component patterns (Fig. 2a, lanes 5 and 6). Corresponding side fractions from the polymerase preparations, containing many proteins, yield largely identical SDS-polyacrylamide gel electrophoresis (PAGE) patterns (Fig. 2b, lanes 7-9). However, several strong bands in the pattern from H₂-grown cells are absent or weak in the pattern of O₂-grown

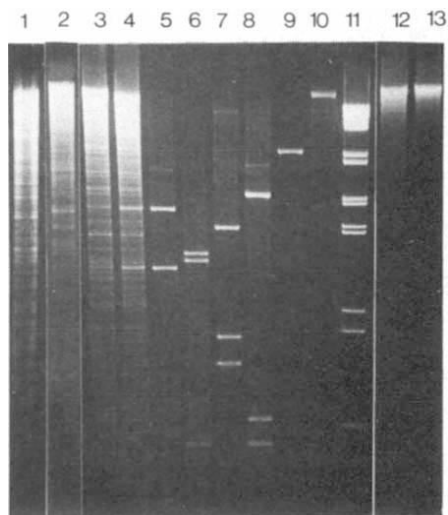


Fig. 1 Agarose gel electrophoresis of restricted and unrestricted DNAs. Lanes 1-4, *Bam*HI-digested DNA of: 1, B6/2; 2, *S. brierleyi*; 3, *S. ambivalens* grown with O₂; 4, *S. ambivalens* grown with H₂. Lanes 5-10, purified plasmid DNA from *S. ambivalens* grown with H₂, digested with: 5, *Bam*HI; 6, *Eco*RI; 7, *Ava*I; 8, *Cla*I; 9, *Hind*III; 10, undigested. Lane 11, bacteriophage T5 DNA digested with *Hind*III as marker; 12, DNA of *S. ambivalens* grown with H₂, undigested; 13, DNA of *S. ambivalens* grown with O₂, undigested.

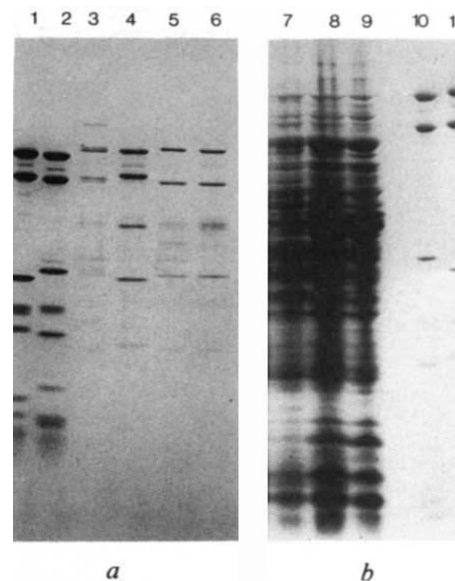


Fig. 2 SDS-PAGE. *a*, DNA-dependent RNA polymerases of: 1 and 11, *S. solfataricus*, strain B12; 2 and 10, *S. acidocaldarius*, DSM 639; 3, strain B6/2 from Beppu, Japan, contaminated; 4, *S. brierleyi*, DSM 1651; 5, *S. ambivalens*, strain L10, grown on O₂+S(0); 6, same, grown on H₂+S(0). Polymerases in lanes 3-6 are from mini preparations. Smaller components are thus invisible. *b*, The supernatant of the polymin P precipitation of the RNA polymerase purification of: 7, *S. ambivalens*, strain L10, grown on O₂+S(0); 8, mixture of 7 and 9; 9, strain L10, grown on H₂+S(0).

cells and vice versa, which would be expected if the two modes of growth result from adaptation of the same organism to the growth requirements, by a switching up and down of the expression of relevant genes.

The protein and DNA fragment patterns of other *Sulfolobus* strains are quite different from the L10 patterns (Figs 1 and 2). All these strains yield DNA-dependent RNA polymerases with clearly different, though similar, component patterns (Fig. 2), which cross-react with each other and with the enzymes from *S. acidocaldarius* and *S. solfataricus* in the immunodiffusion assay of Ouchterlony⁸ that employs an antibody against the polymerase from *S. acidocaldarius* (Fig. 3). This is characteristic of members of the same family. Thus, the strains must be considered as representatives of different species, possibly different genera, of the *Sulfolobus* family (Sulfolobaceae). (A formal description of the novel species will be given elsewhere.) Because of the extent of the differences, a re-evaluation of the phylogeny of the order Sulfolobales, introducing several genera, may be eventually required.

Autotrophic growth by sulphur reduction, in continental solfataric biotopes, so far only known for *Thermoproteus* sp.⁹, is thus also found in Sulfolobales. Sulfolobales and Thermoproteales are phylogenetically related and appear as the

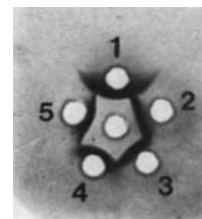


Fig. 3 In-gel immunodiffusion test according to Ouchterlony⁸ with purified γ -globulin directed against DNA-dependent RNA polymerase of *S. acidocaldarius* in centre hole, and the DNA-dependent RNA polymerases of: 1, *S. acidocaldarius*, DSM 639; 2, *S. solfataricus*, DSM 1616; 3, *S. ambivalens*, strain L10; 4, obligatorily autotrophic *S. sp.* strain, B6/2 from Beppu, Japan; 5, *S. brierleyi*, DSM 1651, in surrounding holes.

second strong branch of the deeply divided third urkingdom, besides that comprising the methanogens and extreme halophiles¹⁰. The Sulfolobales may well have originated from ancestors resembling the sulphur reducing Thermoproteales in the energy conservation made, and may have acquired the ability to live by sulphur oxidation later, after oxygen became available in the biosphere.

The amplification of two *Bam*HI restriction fragments in the pattern of the DNA from H₂-grown L10 cells (Fig. 1, lanes 1–4; Fig. 2a, lanes 1–6) suggests that a plasmid is involved. In fact, a second and minor band migrating in front of the diffuse major band of the sheared chromosomal DNA appears more than 5-fold stronger in the pattern of uncut DNA from H₂- than that from O₂-grown cells. The plasmid was purified by a procedure modified from Birnboim and Doly¹¹ involving alkaline denaturation of the chromosomal DNA. As shown by electron microscopy, the product consists mainly of closed circular DNA (not shown). It consists of a major component yielding the two *Bam*HI restriction fragments amplified in cells grown on H₂ and S(0), and a minor component that appears to be a second plasmid (Fig. 1, lane 5). The size of the major plasmid was estimated to be ~7,700 base pairs, that of the minor plasmid 6,500 bp.

Southern hybridization of nick-translated plasmid DNA from *S. ambivalens* to *Bam*HI-digested DNAs from *S. ambivalens*, *S.*

brierleyi, DSM 1651 and the obligatory autotroph *S. sp.*, B6/2 from Beppu, Japan, showed that the plasmid hybridizes only to itself and thus has homology neither with chromosomal DNA from *S. ambivalens* nor with the DNAs of these strains, which do not have the capacity to grow autotrophically via sulphur reduction (data not shown).

At least part of the difference in the protein patterns between H₂- and O₂-grown L10 cells (Fig. 2b, lanes 7 and 9) may be because of the higher copy number of the plasmid, which could harbour genes involved in sulphur reduction. The control of the proteins that appear in O₂- but not in H₂-grown cells must, however, have another basis. Thus different control systems may be operating, supplementing the few models that allow analysis of gene expression in this group of archaeobacteria. It remains to be shown whether plasmids are also related to growth via sulphur reduction of other *Sulfolobus* isolates.

If the plasmid pSL10 suffices to encode the capacity for growth with S(0), H₂ and CO₂, it should be an excellent candidate for a *Sulfolobus* cloning vector.

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Separate sodium and calcium spikes in the same axon

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Aglantha digitale is a jellyfish (order Hydromedusae) capable of two distinct kinds of locomotion; 'slow' swimming which is generated endogenously and is used in fishing behaviour, and 'fast' swimming which is evoked by predators and serves for escape. Both forms of swimming are produced by contraction of the bell-shaped body wall and expulsion of a jet of water from an opening at the base of the animal. During slow swimming, the contractions are weak and the animal moves about 15 mm, roughly one body length, but during a fast swim there is a more violent contraction which can propel the animal five times as far^{1,2}. Both forms of contraction depend on impulses in the eight giant motor axons that synapse directly with the muscle sheet making up the inner surface of the body wall^{3,4}. We report here that the giant motor axons are able to mediate both kinds of activity because they can conduct two different sorts of impulse. Fast swimming requires a rapidly conducted Na⁺-dependent action potential whereas slow swimming depends on a low amplitude Ca²⁺ 'spike'. This is the first report of an axon capable of two kinds of impulse propagation and it provides a physiological function for low potential Ca²⁺ activation⁵.

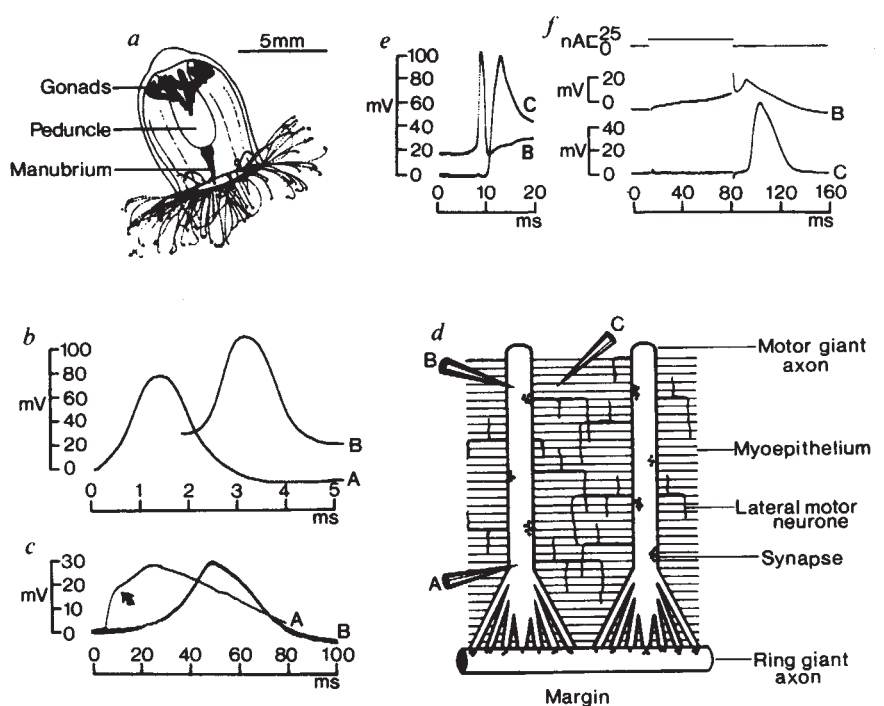
Specimens of *Aglantha* (Fig. 1a) were taken from surface water at the dock of Friday Harbor Laboratories, Washington, United States and anaesthetized in a solution of 1:10 isotonic MgCl₂ and seawater. The apex of the bell was removed and the cylinder of tissue which resulted was slit along one side to

produce a flat sheet. This was turned muscle side up, pinned to a transparent Sylgard platform with spines from the cactus *Opuntia*, washed with seawater and allowed to recover. Most preparations began to give short bursts of swimming activity after ~5 min. Figure 1d (not to scale) shows the innervation of the muscle sheet (myoepithelium) and the major recording sites described in the present study. The myoepithelium is divided into octants by the giant motor axons. In animals 15–20 mm long, the octants are about 2 mm wide; the motor axons are 25–40 µm in diameter and can be seen easily at ×50 magnification by transmitted light. The micropipettes used for intracellular recording had resistances of 70–100 MΩ. All experiments were carried out at 10–13 °C.

Pinned preparations perform spontaneous slow-swimming sequences which resemble the normal activity of the free-swimming animal. A sequence generally consists of 5–10 contractions at ~0.5 Hz and sequences appear at intervals of 2–5 min. Fast-swimming movements, which resemble normal escape behaviour, can be elicited by stimulating the body wall electrically. They are strong, uniform contractions of the whole muscle sheet, in contrast to the slow contractions which are not only weaker but vary in strength along each octant. There was an increased tendency for fast swimming during one of the regular slow-swimming cycles in both pinned and free animals.

Stimuli which initiate fast-swimming movements in a pinned preparation elicit a short burst of action potentials in the 'ring giant' axon (Fig. 1d) that runs around the margin at the base of the animal⁴. These impulses evoke postsynaptic depolarizing events in the giant motor axon which summate to give an overshooting action potential. The action potential is Na⁺ dependent (it is abolished in 10% Na⁺ seawater⁴) and is conducted towards the apex of the bell at a velocity of 1–4 m s⁻¹ (ref. 4 and see Fig. 1b). Events of much smaller amplitude drive slow swimming. In recordings from giant motor axons near their origin at the margin, the slow-swim spike is visible on top of a single postsynaptic potential (Fig. 1c, trace A). This excitatory postsynaptic potential is assumed to represent input from

Fig. 1 Electrophysiology of swimming in *Aglantha*. **a**, Specimen of *Aglantha digitale*. The animal is in a relaxed state at the end of a slow-swimming cycle. The gonads, peduncle and manubrium can be seen through the transparent bell-shaped body wall. Many fine tentacles extend from the margin at the base of the bell. The margin also contains the neurones responsible for generating slow swimming. The giant motor axons that are the subject of the present report run radially from the margin to a point just short of the gonads. They are $\sim 40\ \mu\text{m}$ in diameter in the specimens used here and lie within the sheet of myoepithelium lining the inner surface of the bell. (From a photograph taken by Claudia Mills.) **b**, Intracellularly recorded action potential from a giant motor axon during an escape swim. The two recording sites (represented by A and B in **d**) were 2.5 mm apart and the action potential was conducted away from the margin. Conduction velocity, $1.4\ \text{m s}^{-1}$. Axon resting potential, $-58\ \text{mV}$. **c**, Low-amplitude spike recorded intracellularly from a motor giant axon during an endogenously generated slow swim; the two recording sites were 6.9 mm apart. The synaptic event in the margin (see arrow in trace A) which gives rise to the slow swim can be isolated by raising the Mg^{2+} level in the bathing medium. Conduction velocity, $0.3\ \text{m s}^{-1}$. Axon resting potential, $-70\ \text{mV}$. **d**, Schematic representation of the different recording sites (A, B and C) described in the text. Traces in the other parts of this figure are labelled A, B or C according to the recording site. The lateral motor neurones shown are electrically coupled to the motor giant axons and serve to increase the spread of excitation across the myoepithelium⁸. **e**, Simultaneous intracellular recordings from giant motor axon (trace B) and the nearby myoepithelium (trace C) during an escape swim. The recording sites were 0.3 mm apart and the synaptic delay was 1.2 ms. The rising and falling phases of trace B and the rising phase of trace C have been retouched. Axon resting potential, $-52\ \text{mV}$; muscle resting potential, $-67\ \text{mV}$. **f**, Simultaneous intracellular recordings from giant motor axon (trace B) and nearby myoepithelium (trace C) during a slow swim. Slow-swim spikes could be recorded from lengths of axon as short as $600\ \mu\text{m}$. They were elicited by injecting depolarizing current pulses (top trace) into the giant motor axon via a bridge circuit and were associated with a typical slow swim response in the muscle (trace C). Axon resting potential, $-50\ \text{mV}$; muscle resting potential, $-70\ \text{mV}$.



presynaptic pacemaker neurones, as in other hydromedusae⁶. It is negligibly small at recording sites farther than about half-way along the axon (the space constant of a $40\text{-}\mu\text{m}$ axon is $\sim 4\ \text{mm}$), and so here the slow-swim spike is seen alone (Fig. 1c, trace B), conducted at a velocity of $\sim 0.25\ \text{m s}^{-1}$. Its amplitude is $27\ \text{mV}$ (s.e. $4\ \text{mV}$; $n = 20$), compared with $95\ \text{mV}$ (s.e. $8\ \text{mV}$; $n = 23$) for the fast-swim action potential; it rises more slowly and lasts about 10 times longer. These differences cannot be attributed to different resting membrane potentials because the resting potential of the axon is unchanged during the two forms of activity (Fig. 2b). Average axon resting potentials were $-63\ \text{mV}$ (s.e. $6\ \text{mV}$; $n = 17$) for slow-swim spiking and $-61\ \text{mV}$ (s.e. $5\ \text{mV}$; $n = 10$) for the fast-swim action potential.

The giant motor axons innervate the muscle sheet both directly along their entire length and indirectly through a system of smaller 'lateral' motor neurones to which they are coupled^{3,7,8}. Synaptic transmission is chemical^{4,8}. We find a significant difference in the amplitude of the postsynaptic events recorded from the myoepithelium during fast ($98\ \text{mV}$, s.e. $8\ \text{mV}$; $n = 20$) or slow ($56\ \text{mV}$, s.e. $6\ \text{mV}$; $n = 25$) swimming movements and this is matched by a difference in rise-time (Fig. 1e, f). The low amplitude and slow rise-time would account for the reduced force of contraction observed during slow compared with fast swimming. In Fig. 1f, the slow-swim spike was generated by injecting depolarizing current into the motor giant axon, but comparable records were obtained during spontaneous activity. Stimulated spikes resembled (mean amplitude $27\ \text{mV}$, s.e. $2\ \text{mV}$; $n = 13$) endogenously generated spikes and produced similar postsynaptic responses in the muscle (mean amplitude $59\ \text{mV}$, s.e. $3\ \text{mV}$; $n = 6$).

Figure 2a shows a propagating slow-swim spike generated in the giant motor axon in response to a brief depolarizing current pulse injected at one of the two recording sites. A small increase in current (see top trace) was sufficient to initiate an overshooting

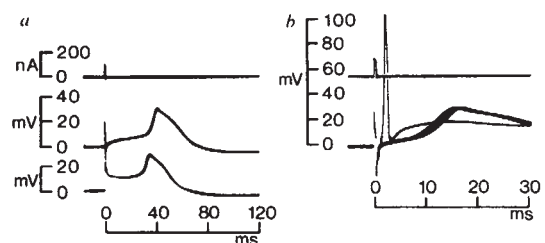
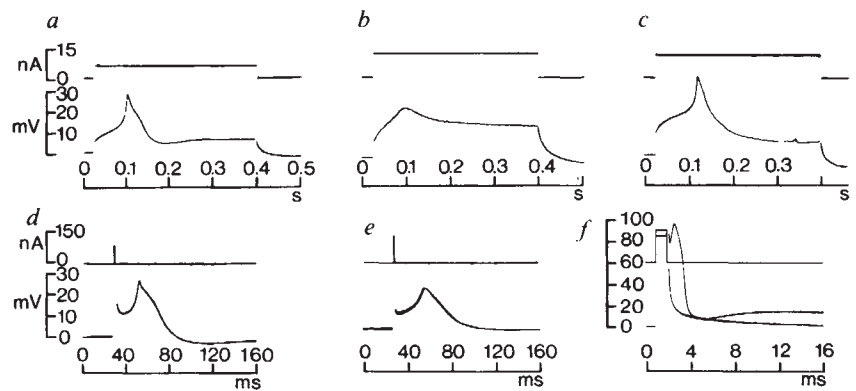


Fig. 2 A slow swim may be elicited by a brief depolarizing current pulse injected into the motor giant axon. **a**, Low-amplitude spike recorded from the motor giant axon in response to a brief depolarizing current pulse (top trace). The current was injected using a bridge circuit so that the membrane potential could be recorded at the site of current injection. The recording sites were $2\ \text{mm}$ apart and the conduction velocity of the slow-swim spike was $\sim 25\%$ of that of the overshooting action potential associated with an escape swim. **b**, A small increase in the intensity of the injected current (see top trace) was sufficient to elicit an overshooting action potential in the same axon. The three superimposed slow-swim spikes were recorded in response to a low level of current injection, while an overshooting action potential appeared when the stimulus intensity was increased. The rising and falling phases of the action potential have been retouched as before. Axon resting potential, $-72\ \text{mV}$.

action potential in the same axon (Fig. 2b). Lower-amplitude but more prolonged depolarizing current pulses also produced the two different forms of action potential even in axons that had been deliberately shortened. Some shortened axons with particularly low resting potentials ($-40\ \text{mV}$) failed to produce a slow-swim spike unless they were hyperpolarized with a conditioning current pulse. We assume this to be because of some voltage-dependent inactivation process. Inactivation would also

Fig. 3 Pharmacological differences between action potentials and slow-swim spikes recorded from the giant motor axon. The slow-swim spike elicited by a depolarizing current pulse (see top traces) in normal seawater (*a*) was reduced by adding 7 mM Mn^{2+} to the bathing medium; there was also an increase in spike threshold (*b*). An increase in external Ca^{2+} from 10 to 28 mM antagonized the effect of Mn^{2+} on the spike amplitude, but the threshold remained high (*c*). The slow-swim spike elicited by a brief depolarizing pulse in normal seawater (*d*) was reduced by seawater containing 0.11 mM nifedipine (*e*). (*f*) Shows that 0.18 mM nifedipine abolished the slow-swim spike (see trace i) but not the overshooting action potential elicited by a slightly increased current (trace ii). Nifedipine was dissolved in ethanol; final levels of ethanol in the bathing solution were $9 \mu\text{l ml}^{-1}$ seawater (*e*) and $15 \mu\text{l ml}^{-1}$ seawater (*f*). Ethanol was tested on other axons at these levels and found to be ineffective. Records *a*–*c* and *d*–*f* were from two different axons. Axon resting potential in *a*, -69 mV ; *d*, -72 mV .



explain why there was no slow-swim spike immediately following the Na^+ action potential. In normal axons, a 15-mV stepwise depolarization (point current injection) generally exceeded the threshold for the slow-swim spike, but the threshold for the fast-swim action potential was significantly higher (about -30 mV).

Endogenously driven slow swimming is blocked by increasing the level of Mg^{2+} in the sea water from 50 to 70 mM. This level of Mg^{2+} does not block synaptic transmission⁸ and instead we find that it acts directly on the slow-swim spike (either spontaneous or evoked) in the giant motor axon. Slow-swim spikes elicited by current injection were also blocked by 7 mM Mn^{2+} (Fig. 3*b*) and this block was reversed by increasing the Ca^{2+} in the bathing medium to 28 mM (Fig. 3*c*). Mn^{2+} (like Mg^{2+}) is known to block Ca^{2+} currents in many different tissues⁹, and so we conclude that the slow-swim spike is a Ca^{2+} -dependent event. This conclusion is supported by experiments with nifedipine, which blocks Ca^{2+} channels in heart and smooth muscle¹⁰; 0.11 mM nifedipine reduced the amplitude of the slow-swim spike (Fig. 3*e*), while 0.18 mM produced a clear block (Fig. 3*f*, trace i). These levels of nifedipine had no effect on the fast overshooting Na^+ -dependent action potential elicited by a somewhat larger current pulse in the same axon (Fig. 3*f*, trace ii).

The Na^+ -dependent action potential is like that found in other axons¹¹; here, as elsewhere, it provides for the rapid transmission of information. The Ca^{2+} -dependent slow-swim spike also has a transmission function, but this is unusual because Ca^{2+} activation is generally associated with more complex data handling. One possible reason for this is that the relatively prolonged period of Ca^{2+} inactivation that follows an increase in Ca^{2+} influx in many cells¹² will limit the rate of firing of Ca^{2+} -dependent spikes. In *Aglantha*, spontaneous slow-swim spiking can occur at rates of up to 5 s^{-1} and so the channels involved must behave rather differently. Ca^{2+} channels that are activated at potentials near the resting potential and which recover rapidly from inactivation, have been found in several different cells^{5,13–17}. In vertebrate neurones, they seem to be responsible for the low-threshold Ca^{2+} spikes recorded from cell bodies in the inferior olive and the thalamus^{18,19}. These spikes resemble the slow-swim spikes we find in *Aglantha*, suggesting that low-potential Ca^{2+} activation may provide a secondary system of spike propagation in vertebrates as well as in invertebrates.

We have shown that slow swimming in *Aglantha* is mediated by a Ca^{2+} -dependent spike, that the escape response depends on a full overshooting Na^+ -dependent action potential and that both regenerative events may be recorded from the same giant motor axon at the same resting potential. They can be evoked independently because the slow-swim spike does not exceed the threshold for the escape response (about -30 mV) and the Ca^{2+} spike is inactivated by depolarization. These two regenerative events have different time courses and evoke different responses in the myoeppithelium. Consequently, they provide a means of

generating both fast and slow contractions in the same muscle sheet.

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Adaptation of *Plasmodium falciparum* to glucose 6-phosphate dehydrogenase-deficient host red cells by production of parasite-encoded enzyme

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There is impressive evidence from geographical data^{1,2}, studies in the field³ and *in vitro* culture work^{4–6} that genetically determined deficiency of glucose 6-phosphate dehydrogenase (G6PD) confers relative protection against the human malaria parasite, *Plasmodium falciparum*. G6PD is encoded by an X-chromosome-linked gene⁷, and protection phenomenon is manifested in heterozygous females who are genetic mosaics but, surprisingly, not in hemizygous males with complete deficiency⁸. We have shown previously that the parasite, when passaged serially through G6PD-deficient

red cells, undergoes adaptive changes that gradually improve its ability to multiply in these deficient cells⁹. To explain the above paradox, we now show that this adaptive process is associated with, and may consist in, the induction of synthesis of a novel G6PD coded by *Plasmodium falciparum*.

When *P. falciparum* is grown in continuous culture, its schizogonic erythrocytic cycle mimics precisely its development in human blood¹⁰. At each cycle, new red blood cells (RBC) are invaded, and these can be appropriately chosen in individual experiments. We have found that, in any particular cycle, the parasite is sensitive to the host cell type and has a memory of the host cell it has inhabited in previous cycles (Fig. 1). Indeed, if aliquots of the same parasite population are made to invade G6PD-normal and G6PD-deficient cells, there is a marked impairment of growth and multiplication in the latter compared with the former (Fig. 1a). In four separate experiments, parasite multiplication expressed as a percentage of control was 43 ± 4 in G6PD-deficient (A^-) RBC, and 47 ± 4 in G6PD-deficient (Mediterranean) RBC. On the other hand, when similar experiments were carried out starting from parasites that had been previously grown through four successive cycles in G6PD-deficient cells, a very slight, not significant, difference was observed (Fig. 1b). We refer to these parasites as being adapted to the G6PD-deficient host cell environment. In four separate experiments, multiplication of these adapted parasites, measured in a fifth cycle in G6PD-deficient (A^-) RBC, was $79 \pm 5\%$ of the multiplication observed on transfer back to G6PD-normal RBC. We have found that adaptation takes place in red cells with either the Mediterranean⁹ or the A^- G6PD deficiency variant (Fig. 1).

We have tested the hypothesis that adaptation could be related to the level of G6PD in the parasitized red cells. *P. falciparum* has been known to produce the related enzyme, 6-phosphogluconate dehydrogenase¹¹, but previous attempts to demonstrate G6PD of parasite origin have been unsuccessful¹², with one exception¹³. In fact, when parasites are grown in normal red cells, we can expect *a priori* that it will be difficult to demonstrate any increase in G6PD activity against the high background of host cell enzyme activity. However, in extracts from adapted cultures in which host cell background G6PD activity is extremely low, we find that G6PD activity is about two to five times higher in parasitized cells than in non-parasitized cells (Table 1). To ensure that this increase in activity was not artefactual, we carried out several control experiments. First, because *P. falciparum* preferentially infects young RBC, we compared the activity of parasitized cells (Table 1d) with that of young, rather than total, RBC (Table 1c). Second, it was conceivable that the extra G6PD activity in parasitized RBC was produced by the parasite rescuing inactive G6PD molecules of host cell origin. This is unlikely, because immunochemical tests have previously failed to detect enzymatically inert G6PD in red cells with the Mediterranean type of G6PD deficiency¹⁴. At any rate, we have ruled out this possibility by taking advantage of the availability of electrophoretically distinct G6PD-deficient variants. Extracts of parasitized G6PD A^- RBC, after

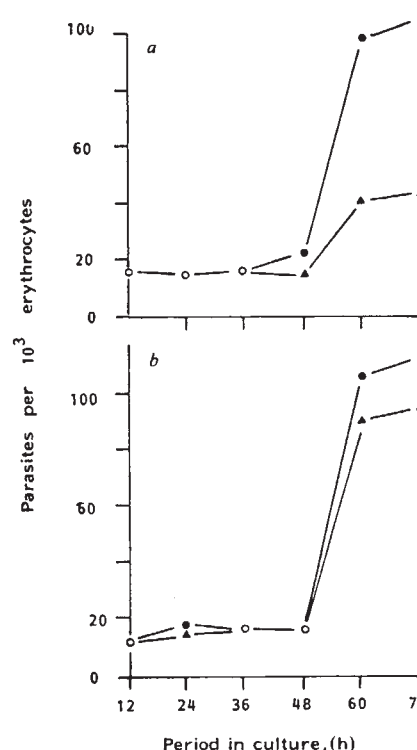


Fig. 1 Adaptation of *P. falciparum* to host red cells of different G6PD type. Parasites were cultured by the method of Trager and Jensen¹⁰. To obtain a synchronous cycle, parasitized RBC were treated with 0.274 M D-sorbitol¹⁸ and then recultured. When the majority of parasites had matured to schizonts, they were enriched by Physiogel¹⁹. The resulting synchronized schizonts were used to infect respective red cells at an initial parasitaemia of 2–3 schizonts per 1,000 RBC. *a*, The inoculum consisted of parasites cultured in normal RBC (G6PD B); *b*, the inoculum consisted of parasites that had been cultured for four cycles in G6PD-deficient (A^-) RBC. ●, Host cells containing normal G6PD; ▲, G6PD-deficient host cells.

adaptation, exhibited two bands of electrophoretically different G6PD activity (Fig. 2, lane 3). By comparison with appropriate controls, we can attribute the faster band to residual host cell enzyme, and we infer that the slower band is parasite specific. (Because the electrophoretic mobility of the parasite enzyme is almost identical with that of normal G6PD B, it is not resolved from the residual host cell enzyme of RBC with G6PD Mediterranean, which is also B-like; Fig. 2, lane 5.) Thus, we conclude that the extra G6PD activity measured in extracts of G6PD-deficient parasitized RBC (last column in Table 1) must result from net synthesis of G6PD by *P. falciparum*.

We next tested whether the expression of parasite-specific G6PD is influenced by the host cell environment. Because, as

Table 1 G6PD activity in red cells parasitized by *P. falciparum* in culture

G6PD type of host RBC	n	<i>b</i> Fresh, total	<i>c</i> Sham-culture, young	<i>d</i> Parasitized	Parasite-specific G6PD activity (<i>d</i> - <i>c</i>)
Normal, B	15	1.79 ± 0.35	3.40 ± 1.12	1.40 ± 0.76	—
Deficient, A^-	11	0.13 ± 0.04	0.097 ± 0.039	0.23 ± 0.10	0.133
Deficient, Med	17	<0.05	0.042 ± 0.012	0.19 ± 0.07	0.148

Values are expressed in IU per 10¹⁰ RBC $\pm$ s.d. G6PD activity was assayed according to WHO¹⁶. In *b*, red cells were sedimented by centrifugation through 86% Percoll (specific gravity 1.055 g ml⁻¹) to effect complete removal of white cells¹⁷. The cells were lysed with 20 μ M NADP and then sonicated for 2 min. The lysate was centrifuged and G6PD activity assayed on the supernatant. In *c*, non-parasitized white-cell-free red cells were incubated in culture medium for the same period as the test culture. The sham-culture was then centrifuged through 90% Percoll (≈ 1.1098 g ml⁻¹) to enrich the young cells. The young cell population was then processed as for *b*. In *d*, after culture, enrichment for RBC containing mature parasites (trophozoites and schizonts) was obtained by centrifugation through 80% Percoll (≈ 1.0970 g ml⁻¹). The resulting cell fractions consisted of 65–85% parasitized cells. These were then washed in phosphate-buffered saline and processed as in *b*.

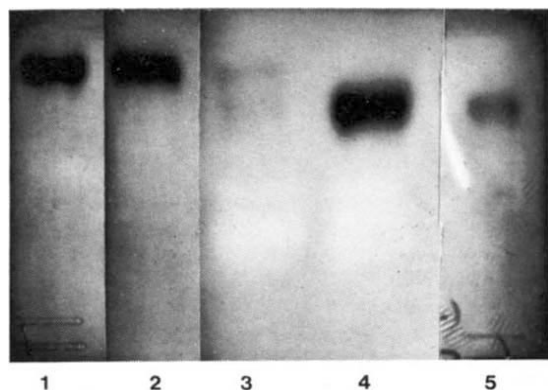


Fig. 2 Demonstration of *P. falciparum*-specific G6PD. Cellophane electrophoresis²⁰ was performed on lysates of uninfected and parasite-enriched erythrocytes. Lysates were prepared as explained in Table 1 and applied to the strips as follows. Lanes 1, 4, uninfected G6PD A and G6PD B red cells, respectively; lane 2, parasitized G6PD A erythrocytes; lane 3, parasitized G6PD A⁻ erythrocytes; lane 5, parasitized G6PD Mediterranean erythrocytes. Electrophoresis of lysates obtained by hypotonic lysis of parasitized G6PD A⁻ red cells, without subsequent disruption of parasites by sonication, showed only the A⁻ band of G6PD activity. The origin is near the bottom.

stated above, the electrophoretic mobility of parasite-encoded G6PD is very similar to that of normal human G6PD B, parasites grown in RBC containing this type of enzyme cannot be used for the test. Therefore, we have grown *P. falciparum* through four cycles in RBC containing an electrophoretically variant G6PD A, which has normal activity. Electrophoresis of extracts of these parasitized red cells failed to show any band other than G6PD type A (Fig. 2, lane 2). Thus, there is no significant production of parasite-specific G6PD when the parasites are adapted to G6PD normal RBC.

We conclude that *P. falciparum* can produce G6PD, implying that it has a gene specifying this enzyme. The expression of this gene, in our experiments, is detectable only when G6PD-deficient RBC are used as host cells. We believe this to be the first example of an adaptive phenomenon in an intracellular parasitic protozoan to a mutant host cell environment which can be interpreted in terms of regulation of a specific parasite gene. This behaviour of *P. falciparum* explains why hemizygous G6PD-deficient males are not protected against the infection and also explains the protection of heterozygous females^{3,15}, for in the latter subjects, *P. falciparum* merozoites emerging from normal RBC have, on the average, an even chance of infecting a normal or a G6PD-deficient cell. In the latter case, the chance to complete successfully the next schizogonic cycle is reduced by ~50% (see Table 1), thus justifying the reduced parasitaemia actually observed. This mechanism of host resistance is made possible by red cell mosaicism, thus illustrating a new biological implication of the unique phenomenon of X-chromosome inactivation.

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Detection *in vivo* of protein-DNA interactions within the *lac* operon of *Escherichia coli*

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Studies of the sequence-specific binding of proteins to DNA have so far relied on *in vitro* experiments using cloned restriction fragments containing the relevant DNA sequences¹⁻⁶. We have applied the genomic sequencing technique of Church and Gilbert⁷ to show that the interactions observed *in vitro* occur *in vivo*. We use this approach to study the binding of regulatory proteins to the *lac* operon *in vivo* and detect changes in the reactivity (inhibition or enhancement) of guanines to methylation by dimethyl sulphate caused by the proximity of proteins to the N-7 atom of these guanines^{1,5}. We can detect the simultaneous binding of the catabolite gene activator protein (CAP) and the Lac repressor to their specific recognition sequences, and following induction of the *lac* operon we observe effects that are related to RNA polymerase binding or RNA elongation. We have successfully used oligonucleotide probes as short as 17 bases to display genomic sequence.

The expression of the *lac* operon of *Escherichia coli* is regulated by two allosteric proteins: the Lac repressor, which binds to the operator and blocks RNA polymerase entry to the promoter, except in the presence of lactose analogues which bind to the repressor, reduce its affinity for the operator and allow the initiation of transcription⁸⁻¹²; and CAP, which, when complexed with cyclic AMP, binds to the promoter and stimulates transcription (intracellular cyclic AMP levels are reduced in the presence of glucose, which accounts for the phenomenon of catabolite repression by reducing CAP binding)¹³⁻¹⁷.

We have investigated these DNA-protein interactions by an application of a genomic sequencing technique⁷. The first step was to methylate guanine bases on the *E. coli* chromosome by exposure of exponentially growing cells to dimethyl sulphate (DMS). Extracted DNA was then digested to completion with a restriction enzyme and cleaved at methylated guanines by piperidine treatment. The denatured DNA fragments thus generated were then separated on a sequencing gel and electrophoretically transferred to a nylon support. The fragments were covalently crosslinked to the nylon by irradiation with ultraviolet light⁷. The sequence of a particular region of the *E. coli* chromosome could then be displayed by hybridizing the nylon support to a strand-specific radioactively labelled probe, and to allow precise positioning of the sites of piperidine cleavage it is necessary to use a probe that abuts the restriction enzyme site closest to the

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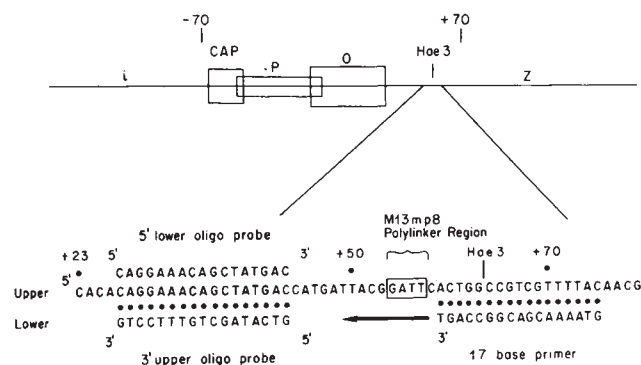


Fig. 1 The *lac* operon control region and the strategy for detection of genomic sequence. Top, the repressor gene (*i*), the CAP binding site, the promoter region (*P*) or RNA polymerase site, and the operator (*O*) are shown. The expanded region shows the wild-type *lac* sequence to which the M13 mp8-lacZ primer and 17-mer probes bind. The bracket indicates the position of the polylinker region of M13 mp8. The two 17-mer probes (obtained from PL Biochemicals) when labelled, display either 5' lower or 3' upper sequence (●) (the data from the 3' upper 17-mer probe are not shown). Synthesis from the 17-nucleotide primer (Collaborative Research) annealed to the single-strand M13 DNA proceeds in the direction of the arrow and is limited to ~100 bases by the concentration of the radioactive nucleotide⁷.

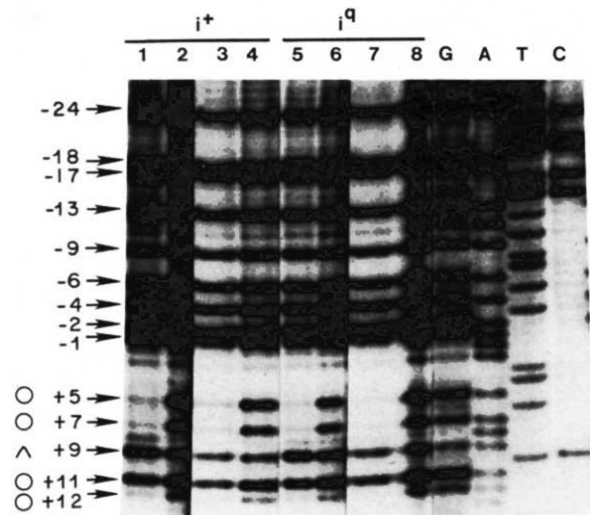


Fig. 2 A genomic sequence of the *lac* operator showing the effects of toluene and various cellular Lac repressor concentrations in the presence and absence of IPTG. Lanes G, A, T and C illustrate the sequence from naked DNA²⁹. Lanes 1-4 are from a wild-type *E. coli* strain, *lacI*⁺, (FMA10, F. Ausubel; W3102, *hsdr Tdr*⁻) and lanes 5-8 correspond to a Lac repressor overproducer, *lacI*^q (JM101)¹⁸. Lanes 1, 2, 5, 6 show DMS treatment of whole cells; lanes 3, 4, 7, 8, reactions with permeabilized cells. Lanes 2, 4, 6, 8 correspond to cells grown in the presence of 1 mM IPTG. The corresponding numbered positions are shown relative to the start of transcription (+1). The symbols ○ and △ indicate protection and enhancement, respectively.

Methods. Intact cells in rich medium were treated with 0.5% DMS for 2 min (same % and time for all subsequent data). The reaction was terminated by dilution in cold medium and lysozyme was added at 4 °C followed by lysis with an SDS-EDTA solution. The genomic DNA was restricted with *Hae*III and 1 µg of piperidine-treated genomic DNA was loaded per lane on a 0.04-cm thick 7 M urea gel⁷. Hybridization with an M13 mp8-lacZ probe was allowed to proceed at 65 °C for 16 h and the filter was washed in 0.5 M Na⁺ at 65 °C followed by eight washes in 0.3 M Na⁺. The filter was exposed for 10 days without an intensifying screen.

region of interest. For our analysis of the *lac* operon which used a *Hae*III site, one probe was generated by copying the vector M13 mp8 (ref. 18) using an mp8-lacZ sequencing primer, as mp8 contains the *lac* promoter and operator DNA ('3' upper', Fig. 1). The other probe was a chemically synthesized 17-nucleotide fragment ('5' lower', Fig. 1).

Figure 2 shows a genomic sequence of the 3' upper strand of the *lac* control region of *E. coli* displayed with the M13 mp8 derived probe. It also shows the results of DMS treatment of *E. coli* strains with the wild-type *lac* genotype, *lacI*⁺ (lanes 1-4) and a Lac repressor overproducer (*lacI*^q) strain¹⁸ (lanes 5-8), both grown in YT media with or without the gratuitous inducer isopropyl thiogalactoside (IPTG). Identical samples were permeabilized with toluene¹⁹ before DMS treatment (Fig. 2, lanes 3, 4, 7, 8). We found that the Lac repressor inhibited the methylation by DMS at positions 5, 7, 11 and 12 and enhanced methylation at the guanine at position 9 (Fig. 2, lanes 1, 3, 5, 7). IPTG abolished these effects (Fig. 2, lanes 2, 4, 6, 8), in agreement with the changes reported *in vitro*⁵. Clearly, DMS enters living cells and there is thus no need for toluene permeabilization. We detected repressor binding at wild-type concentrations (10 molecules of Lac repressor per cell¹⁰).

We next monitored the binding of CAP to the Lac promoter by using strains carrying wild-type CAP genes on plasmids containing either their own promoter or one derived from pBR322 (ref. 20). Figure 3 compares cells grown in minimal

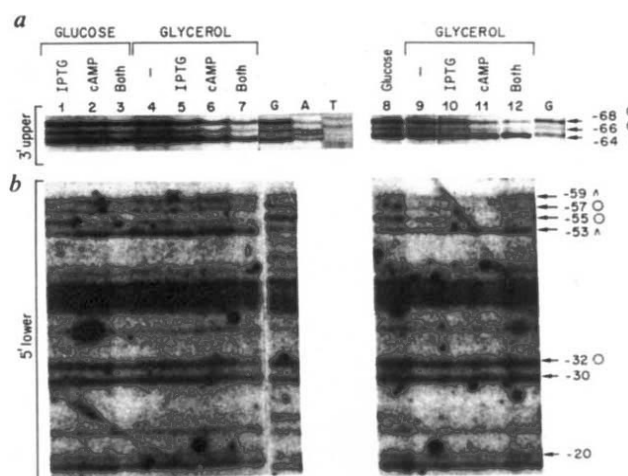


Fig. 3 Detection of cyclic AMP-CAP binding to the Lac promoter *in vivo* and the use of an oligonucleotide probe to display genomic sequence. Experiments using strain PB5/pHA5 (*CRP39*, $\Delta gal165$ *thi*) in minimal media. Lanes G, A and T are chemical reactions on naked DNA specific for guanine, adenine and thymine, respectively²⁹. Strain PB5/pHA5 was grown, concentrated in the respective medium, treated with DMS and diluted 4x in cold Dulbecco's phosphate-buffered saline (PBS). The filter was prepared as in Fig. 2 and the upper strand displayed with an M13 mp8-lacZ probe. The right half of a shows a similar experiment to that shown on the left except that samples were reacted with DMS at 37 °C without concentration by centrifugation. Two strains are compared: lanes 8 (glucose alone) and 12 are pp47/pHA7 (pBR322 promoter)²⁰; lanes 9-11 are DMS reactions on strain PB5/pHA5 (wild-type promoter). Lane G illustrates DMS reaction with naked genomic DNA from PB5/pHA5. b illustrates the use of an end-labelled 17-nucleotide probe. The strategy for using the oligonucleotide probe is given in Fig. 1. All the lanes are aligned with the corresponding lanes and conditions were as in a. The samples and filter were prepared as described in the legends to Figs 2 and 3a. The 5' lower oligonucleotide probe (100-200 pmol) was end-labelled by kinase treatment and purified as for the M13 probes⁷. Hybridization was performed at 45 °C in 1 M Na⁺ rather than 0.7 M (ref. 7), with two low-stringency washes at 45 °C, 0.4 Na⁺ and eight high-stringency washes at 0.2 M Na⁺. The symbols ○ and △ indicate protection and enhancement, respectively. The 17-mer-probed filter was exposed with an intensifying screen for 10 days at -70 °C.

media plus glucose (catabolite repressed) or glycerol (a catabolite that produces higher cellular levels of cyclic AMP than does glucose^{13,21}), with or without cyclic AMP and IPTG.

Protections at positions -68 and -66 and enhancement at guanine -64, compared with naked DNA, are apparent in Fig. 3a, most strongly with glycerol plus IPTG alone (lane 6) or with cyclic AMP (lane 7). This is CAP binding and these changes coincide with those observed *in vitro* by Majors^{22,23}. Such changes do not seem to be under catabolite repression (Fig. 3a, lane 1). Moreover, growth on glycerol allows for levels of cyclic AMP high enough for CAP binding (Fig. 3a, lanes 4, 5). Treatment at 37 °C with DMS in the growth flask rather than after

centrifugation, to avoid any physiological shock, increased the effect at each guanine residue (Fig. 3a, compare lanes 6, 7 with 11, 12). Figure 3b displays the lower strand DMS pattern obtained using the 5' lower 17-mer probe shown in Fig. 1; each lane is aligned with the corresponding condition in Fig. 3a. The interaction of CAP with the 5' lower strand occurs through enhancements at positions -53 and -59 and protection from methylation at -55 and -57 (Fig. 3b, lanes 2-7 and 9-12, respectively; compare with lane G). These changes do not occur in the presence of glucose without cyclic AMP (Fig. 3b, lanes 1, 8).

Our data from both strands confirm the interaction of CAP *in vivo* with the centre of the 2-fold symmetrical canonical sequence, 5'GTGAG---CTCAC 3' (Fig. 4d), of the promoter site^{17,24}. Additionally, it reaffirms the enhanced reactivity to DMS at positions -64 (3' upper), -53 and -59 (5' lower)^{23,24}, which have previously been neglected in model building of the protein-DNA complex²⁵.

A densitometric analysis was performed on the upper-strand filters. Figure 4 plots the logarithms of the ratios of intensities (control/experimental) at the guanine residues of the upper strand. Figure 4a shows the effects of different growth conditions on the changes in methylation at the CAP site, upper strand, while Fig. 4b shows densitometric data for growth conditions lacking IPTG. These experiments illustrate the binding of Lac repressor to the operator and the co-occupancy of the control region by repressor and CAP²⁶ (Fig. 4a, b, B, D, F). Induction by 1 mM IPTG abolishes repressor-specific protection, replacing it with enhancement at positions -1 to +12 (Fig. 4c). The effects at positions -18, -13, -6 and -1 (Fig. 4c, d) and on the 5' lower strand at position -32 (Fig. 3b, lanes 3, 7) coincide with the prominent changes documented *in vitro* by Johnsrud² for RNA polymerase binding. The quantitative changes at these guanines are smaller than *in vitro*, but this is to be expected, as the *in vivo* occupancy should be lower. The series of enhancements following -1 have no analogous *in vitro* protection experiments and probably reflect an active transcriptional complex engaged in RNA chain elongation.

We have presented here a rapid and conclusive method for detection of protein-DNA interactions *in vivo*. Our application of genomic sequencing could be further extended if used in conjunction with photochemical protection experiments²⁷ or the photochemistry devised by Becker and Wang²⁸.

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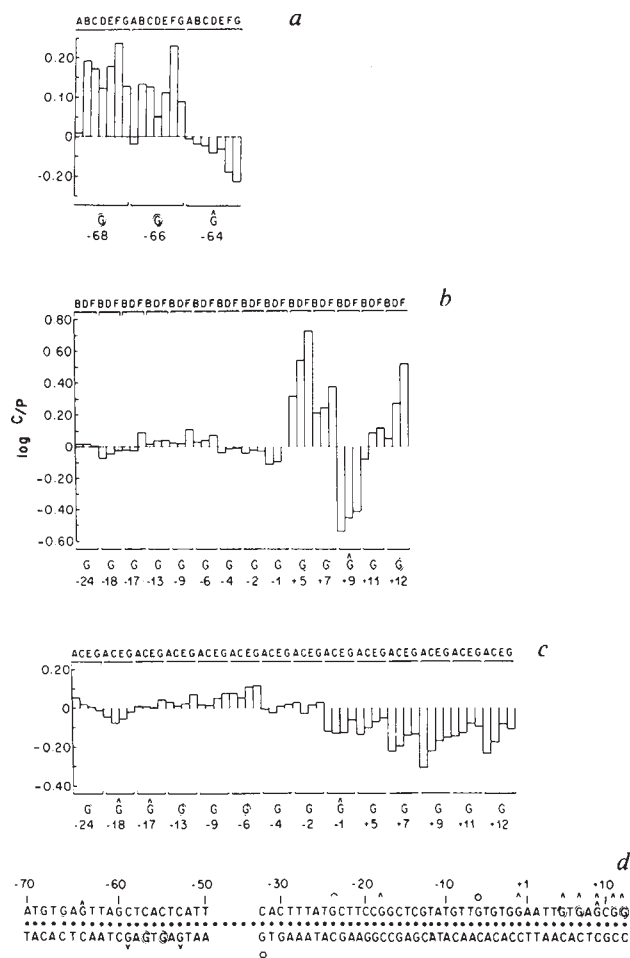


Fig. 4 Quantitation by densitometry of the reactivity of each guanine residue to DMS. The densitometric data correspond to the upper-strand sequence of Fig. 1. Autoradiograms were scanned by an Ortec 4310 densitometer to measure the intensity of each band. The data are expressed as the log of the ratio C/P , where C is the measured intensity of the band in the control lane (naked DNA) and P is the intensity of that band in each respective *in vivo* sample⁵; a positive value corresponds to protection and a negative value enhancement. The abscissa aligns each growth condition with a guanine residue and its numerical position in the sequence. **a**, **b**, **c** Summarize our data for CAP, the Lac repressor and the active transcriptional complex, respectively. Not all of the data from which these graphs were derived are shown. **A**, Glucose (1%) and IPTG (1×10^{-3} M); **B**, glucose and cyclic AMP (5×10^{-3} M); **C**, glucose, IPTG and cyclic AMP; **D**, glycerol (1%); **E**, glycerol and IPTG; **F**, glycerol and cyclic AMP; **G**, glycerol, IPTG, and cyclic AMP. The protection (○) and enhancement (△) on the Gs of the abscissa summarize *in vitro* data for CAP (**a**)^{22,23}, repressor (**b**)^{1,5} and RNA polymerase (**c**)². **d**, The primary sequence of the Lac promoter and operator with a complete summary of the *in vivo* protections (○) and enhancements (△). Data from transcriptionally inactive promoters (**a**, **b**) are placed directly on the sequence and that for active promoters (**c**) above the sequence.

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Cell-type-specific contacts to immunoglobulin enhancers in nuclei

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The introns separating the variable and constant regions of active immunoglobulin genes contain tissue-specific transcriptional enhancer elements¹⁻³, DNA segments which act in *cis* in an orientation- and distance-independent (up to a few kilobases (kb)) manner to enhance transcription initiation at adjacent promoters⁴⁻⁹. The immunoglobulin heavy-chain enhancer is active only in lymphoid cells: in transfection assays it is capable of controlling in *cis* transcription from the simian virus 40 (SV40) T-antigen, rabbit β -globin and immunoglobulin gene promoters up to at least 2 kb away¹⁻³. Genetic deletion analysis suggests that a region of as few as 140 base pairs (bp) is sufficient for the enhancement effect^{1,2}. These functional characteristics and DNA sequences are conserved between mouse and man¹⁰⁻¹⁵. However, it is not known whether tissue-specific proteins bind to the enhancer. Proteins that interact with DNA at specific sequences can prevent or enhance the reactions of individual guanines or adenines with dimethyl sulphate (DMS)¹⁶, and this property has been used to display the DNA contacts of various regulatory proteins¹⁶⁻²⁶. Here we apply this DMS strategy in experiments involving single-copy genes within intact mammalian nuclei using genomic sequencing²⁷.

Nuclei were isolated from tissue culture cells and treated with DMS at 20 °C (Fig. 2A legend). The DNA was then isolated, cleaved with *Eco*RI, electrophoresed, after appropriate chemical treatment, on a denaturing sequencing gel, and the lanes of DNA were electrophoretically transferred and crosslinked to nylon membranes using ultraviolet light. Hybridization with a short single-stranded probe (Fig. 1) complementary to the terminal 110 nucleotides of one strand produced the image of a DNA sequencing ladder extending from the 3' or 5' end of the appropriate *Eco*RI site in the genome.

Figure 2A shows the genomic sequence pattern for the upper strand of the enhancer region of J558L, an immunoglobulin A (IgA) messenger RNA-producing myeloma, M104E, an IgM mRNA-producing myeloma, and L-cells. Figure 2A, lanes f, g, display the pattern produced by DMS treatment of J55L nuclei for 1 and 2 min, respectively. A band is present for every guanine (or cluster of guanines) expected from the known sequence, from positions 483 to 551; bands do not appear at any other position. The band at the position of the open circle in Fig. 2A, lane g (nuclei), is light (protected from DMS) relative to the band at the corresponding position in lane h (isolated DNA). The band just below this is darker (enhanced DMS reactivity) in the myeloma nuclei relative to the isolated DNA. Figure 4 shows the positions of these effects in the enhancer sequence, at guanine 404 (protected) and at the guanine doublet 406-407 (enhanced); the 406-407 doublet was not resolved. The IgM-producing MOPC-104E myeloma ascites tumour displays a similar pattern of protected and enhanced guanines (Fig. 2A,

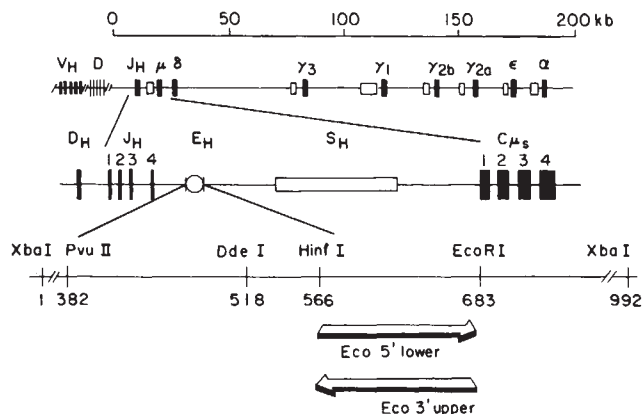


Fig. 1 Immunoglobulin heavy-chain gene structure and DNA probe strategy. The top line displays an overview of the locus, including variable, diversity and joining regions (respectively V_H , D_H , J_H) and eight constant-region exon clusters (μ - α , dark boxes). The enhancer region (E_H , open circle) is shown relative to flanking exons (D_H , J_H , C_H , dark boxes) and switch regions (S_H , open boxes) present in the unrearranged germline configurations. Beneath it is an expansion of the enhancer region showing the positions of restriction sites used relative to the *Xba*I site. The sense of the single-stranded ³²P-labelled DNA probes is indicated by large arrows pointing 5' to 3'. The probe names refer to the type of sequence they will produce. For example, *Eco* 3' upper will produce the sequence ladder expected of 3' end-labelling the *Eco*RI site. The 112-bp *Eco*RI/*Hinf*I fragment cloned in both orientations into *Sma*I-cut M13 mp8 phage vector provided the single-stranded templates for probe syntheses as described elsewhere²⁷.

lanes j, k), while nuclei from fibroblastic non-lymphoid L-cells²⁸ do not show this effect (Fig. 2A, lanes n, o).

Figure 2B shows the opposite strand obtained by reprobng the same membrane with the *Eco* 5' lower probe. No effects were seen in the region near 404-407 on the other strand. However, 125 bp away, guanine 530 shows enhanced reactivity relative to isolated DNA or L-cell nuclei. Three guanines are indicated in Fig. 2B (positions 531, 533, 534) which showed DMS protection in Fig. 3A (lanes a, d, f, m, n) and Fig. 3D (lanes a, e-h). The two clusters of affected bases, centred about positions 404 and 533 (Fig. 4), show similar (inverted) sequence and protection patterns.

The two myelomas J558L and MOPC-104E produce high levels of immunoglobulin heavy-chain mRNA. There is only one allele of the enhancer in MOPC-104E; the other is deleted (Y. Kurosawa and S.T., unpublished). In J558L, we see the average of two allelic enhancers in two different chromosomal locations; one is producing heavy-chain mRNA, the other has been translocated to within the *c-myc* oncogene 5' noncoding region²⁹. In L-cells, both enhancer alleles should be in the germline configuration.

The effects observed cannot reflect the inherent variations in the DMS reactivity of guanines in double-stranded DNA or variations due to DNA sequence polymorphisms or somatic mutations, because DMS and piperidine treatments were performed on isolated DNA from each cell line in the same type of buffer as used for the nuclei (Fig. 2A, B, lanes a, h, l). Could the changes in DMS reactivity be the consequence of transcription opening up short stretches of single-stranded DNA? Certain guanines, such as the middle guanine of a 5' GGA 3' sequence, have a weak reaction in native DNA which can be increased in denatured DNA²⁰. The RNA polymerase increases the reaction of a guanine in the *lac* promoter by this mechanism²⁰. Reactions in denaturing formamide for the enhancer guanines assayed here³⁶ result in slight enhancements relative to native DNA at four 5' GGA 3' sequences, but not at the sites we find enhanced in nuclei. Thus, DNA secondary structural changes do not explain these effects.

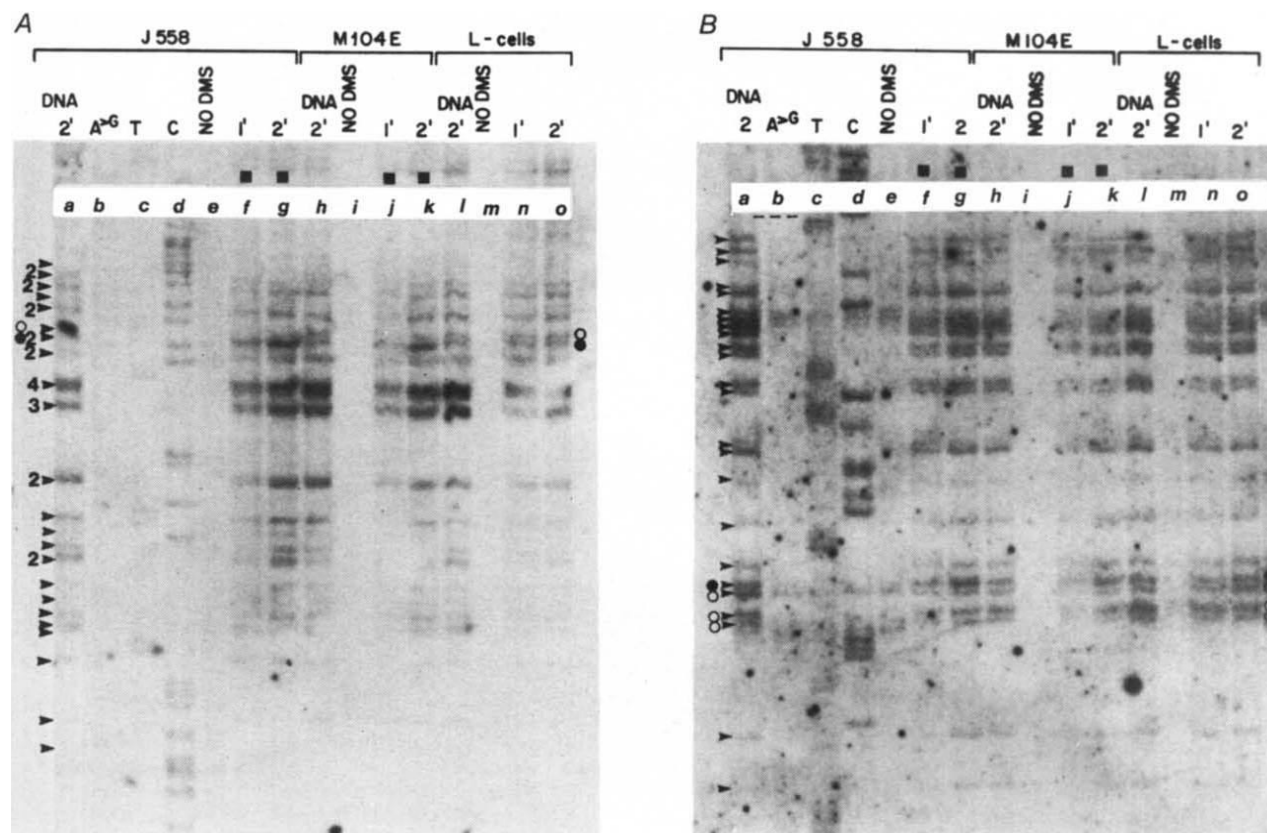


Fig. 2 **A**, DMS reactions in myeloma and fibroblast nuclei. Lanes *a-g*, DNA from J558L myeloma cells¹ (α , λ producers); lanes *h-k*, DNA from MOPC-104E myeloma cells³² (μ , λ producers); lanes *l-o*, DNA from L-cells (*tk*⁻ *aprt*⁻)²⁸ (no immunoglobulin production). Nuclei were prepared by Dounce homogenization with the loose pestle at 4 °C in 250 mM sucrose, 60 mM KCl, 15 mM NaCl, 3 mM MgCl₂, 15 mM Tris pH 7.5, 0.5 mM β -mercaptoethanol, 20 μ M EGTA, 0.05% Triton X-100, followed by addition of 2 M sucrose to a final concentration of 500 mM and pelleting at 800g for 5 min. The loose nuclear pellet is easily resuspended in the same buffer lacking Triton, transferred away from the lower packed cell and debris pellet, then repelleted and resuspended at 10⁸ nuclei ml⁻¹. Tris buffer and β -mercaptoethanol do not interfere with the DNA reaction with DMS. In lanes *f, j, n*, nuclei were incubated with 0.5% DMS (50 mM) at 20 °C for 1 min. In lanes *g, k, o*, nuclei were incubated with 0.5% DMS at 20 °C for 2 min. Lanes *a, h, l* are control DMS reactions on isolated DNA. In lanes *e, i, m*, control nuclei were incubated without DMS at 20 °C for 2 min. Lanes *b-d* are A>G, T and C reactions³³⁻³⁵, respectively, using isolated J558L DNA. The reactions were analysed by cleavage with *Eco*RI enzyme, ethanol precipitation and the direct genomic sequencing procedure²⁷, loading 15–30 μ g of DNA per lane. The lanes were transferred and crosslinked to a nylon membrane and probed with the *Eco* 3' upper probe described in Fig. 1. The arrows indicate positions of all guanines. Positions 1–190 (see Figs 1, 5) were assayed. Shown here are 483 (topmost arrow) to 551 (bottom). Clusters of two, three or four guanines which were not well resolved are indicated by numbers beside the arrows. Guanines are indicated which are either protected (○) or enhanced (●) in a lymphoid-specific fashion. ■, Those lanes where these altered reactivities are observed. MOPC-104E has only a single copy per cell of the region displayed; we detect 1 fg (2 $\times$ 10⁴ molecules) of probe complementary DNA in each band (there are 6 $\times$ 10⁻¹⁸ molecules per lane). **B**, After autoradiography, the membrane used in **A** was stripped of the first probe and reprobed with the *Eco* 5' lower probe. The lanes and symbols are as in **A**. The topmost arrow indicates position 384, the bottom-most indicates position 494.

The dark bands interpreted as enhanced guanines are not caused by crossreacting or contaminating restriction fragments of the appropriate lengths, as no enhanced bands of equal length appear on the opposite strand. Also, *Eco*RI low-specificity nicking, cell-type-specific endogenous nuclease or depurination activities are ruled out by the omission of DMS from the incubations in Fig. 2A, B (lanes *e, i, m*). Figure 3A, lanes *c, e, h, k*, which omits the piperidine step, demonstrates controls for any DMS-activated cell-type-specific nucleases. The tissue-specific effects on DMS reactivity are best explained by molecules (proteins) making contacts near guanine N-7 atoms in the major groove of the enhancer DNA. These experiments do not prove that the binding of such molecules is the cause, rather than an effect, of the transcriptional activity.

To provide additional controls and as a step towards isolating the molecules responsible for the protection patterns, the stabilities of the putative DNA-protein interactions were monitored as a function of buffer composition and ionic strength. Figure 3A shows that the cell-type-specific patterns at guanines 530–534 in the J558L myeloma are resistant to modest alterations in the DMS reaction protocol, for example a fourfold higher

Tris concentration (lanes *a-o*), 0 °C (lanes *a, b*), 150 mM monovalent cation concentration (lanes *f, g*) or 6 mM EGTA (lanes *m-o*). The controls in these experiments use the non-immunoglobulin-expressing³⁰ Friend virus-transformed MEL³¹ erythroleukaemic cells, which are non-adherent and closer than L-cells in developmental lineage to lymphoid cells. The interaction responsible for the altered DMS reactivity at the lower site is sensitive to 250 mM monovalent cation concentration (Fig. 3A, lane *i*) and higher (lane *l*). The upper site at 404–407 shows the same sensitivity on reprobing the membrane in Fig. 3B. The striking difference between the enhanced and the protected guanines visible at 75 and 150 mM (Fig. 3A, B, *d, f, m, n*) is eliminated at 250 and 500 mM salt (Fig. 3A, B, lanes *i, l*). Note the similarity of the myeloma and erythroid patterns at high salt concentrations (Fig. 3A, B, lanes *i, j*). Figure 3C shows that small shifts in the lymphoid pattern of residues 404–407 towards the pattern seen with isolated DNA do not begin until about 225 mM salt. The protection pattern of residues 530–534 is lost at the same concentrations on the other strand (not shown). Some loss can occasionally be seen at both sites at concentrations as low as 75 mM (Fig. 3D, lane *e*). The patterns are clearest in

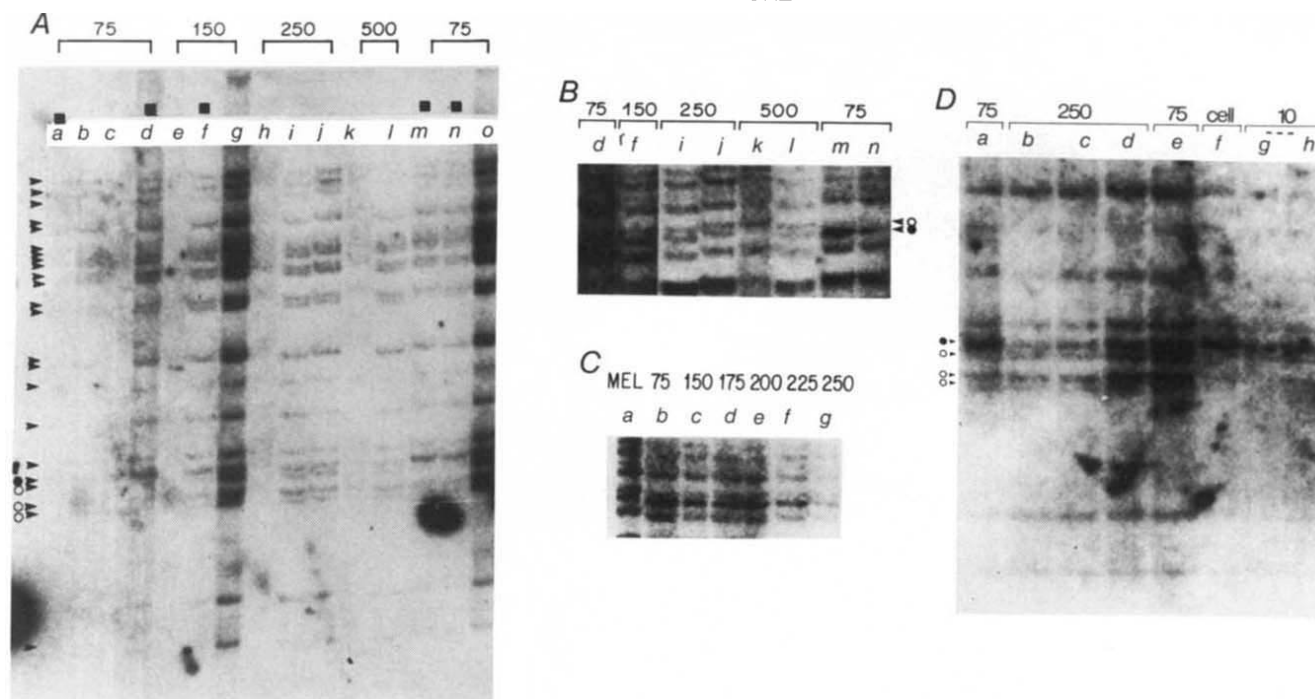


Fig. 3 **A**, Effect of NaCl on DMS reaction patterns in lymphoid and erythroid nuclei. Nuclei isolation conditions were as for Fig. 2. Lanes *a, b*, nuclei were incubated with 0.5% DMS at 4°C for 8 min. Lanes *c-o*, nuclei were incubated with 0.5% DMS at 20°C for 4 min. Lanes *a, c-f, h-i, k-n*, DNA from J558L cell nuclei. Lanes *b, g, j, o*, DNA from MEL-D2-745-JG1-PC4 erythroleukaemic³¹ cell nuclei. Lanes *c, e, h, k*, controls where piperidine cleavage at modified guanine bases was omitted. The Na⁺ plus K⁺ concentrations were as follows: 75 mM in lanes *a-d* and *m-o*; 150 mM in lanes *e-g*; 250 mM in lanes *h-j*; 500 mM in lanes *k-l*. The basic reaction buffer (75 mM Na⁺ plus K⁺) consisted of (in mM) 250 sucrose, 60 KCl, 15 NaCl, 3 MgCl₂, 60 Tris pH 8.2, 0.5 β-mercaptoethanol and 20 μM EGTA. Other solutions contained higher NaCl concentrations. The solutions used in lanes *m-o* contained 6 mM EGTA. **B**, After autoradiography, the membrane was stripped of the first probe and reprobed with the *Eco* 3' upper probe. The lanes and symbols are as for **A**. The enhancement/protection symbols are at positions 404-407. **C**, As for **A** and **B**, adjusting only NaCl concentrations to achieve total monovalent cation concentrations indicated at the head of each lane (in mM). The site at 404-407 is shown using the *Eco* 3' upper probe. **D**, The lower strand of guanines near site 531-534, showing effects of very low salt and transient high salt treatment. DMS reactions for lanes *a* and *e* were done in standard (75 mM) conditions as in previous figures. Lane *b*, DMS reaction at 250 mM salt; lane *c*, at 75 mM after dilution from 250 mM; lane *d*, at 75 mM after washing nuclei at 250 mM; lane *f*, DMS reaction on cells in growth medium³⁶. Lanes *g, h*, nuclei at 10 mM NaCl, 0.05% Triton X-100 with 10 mM Tris-HCl pH 7.5 and 50 mM Tris pH 8.2, respectively.

very low salt, 10 mM (lanes *g, h*) and these are comparable to what is seen in whole cells treated with DMS³⁶. The salt suppression of the tissue-specific reaction patterns observed might be due directly to titration of electrostatic interactions or indirectly to changes in DNA conformation or to the major release of unrelated chromosomal proteins. In any case, these data support our suggestion that the two sites have similar properties, including salt elution.

The similarities in DNA sequence and DMS reactivity pattern between the two putative binding sites noted above could represent binding by a single recognition protein. If this protein were necessary for a lymphoid-specific function, we would predict that similar DNA sequences would be found in other known immunoglobulin enhancers and homologous regions in other species. A human heavy-chain enhancer which functions in mouse lymphoid cells lies within a 279-bp *AluI* fragment¹³ and contains two homologous sequences at positions similar to those in the mouse gene (Fig. 5). A mouse κ enhancer is contained on a 475-bp *AluI* fragment between the κ joining (J_{κ}) and constant (C_{κ}) region. Within this region, a 130-bp stretch is conserved among mice, rabbits and man¹¹, and contains two homologous 10-bp sequences in all three species. Outside this region there are two further matches. Figure 5 derives a consensus sequence, GCCAGGTGGC, from these comparisons.

We interpret the broad variety of deletion studies^{1,2,10,13} as showing that other sequences as well as the consensus sequences are necessary for enhancer action. A single 10-bp immunoglobulin consensus element alone is probably not sufficient for enhancement because single examples of similar sequences (Fig. 5) occur in pBR322 and SV40, while these vectors in transfection assays have little or no lymphoid-specific transcription back-

ground. If the 10-bp element does contribute to lymphoid-specific transcriptional enhancement, one must be cautious in interpreting the deletion data. For example, the 140-bp *PvuII/DdeI* enhancer fragment¹ inserted 80 bp from the pBR322 match could represent an unanticipated duplication of the 10-bp elements synergistically producing some enhancement activity.

The significance of the reiterated consensus sequence can be tested by examining the DMS reactivity of the other immunoglobulin enhancers in various cell types and by analysing the effects of point mutations on DNA protection/enhancement patterns and transcription levels after transfection. The DMS reactivity patterns provide assays for isolating enhancer binding molecules.

In conclusion, we have detected a factor that binds to an enhancer element in a tissue- and sequence-specific fashion.



Fig. 4 Summary of positions of G nucleotides with altered DMS reactivities within the immunoglobulin heavy-chain enhancer region. Altered DMS reactivities of guanines specific for lymphoid nuclei are indicated for protected (○) and enhanced (●), relative to isolated DNA. Thin lines indicate homologies to viral enhancer core sequences³⁸ noted in previous work^{1,2,10-15}. Numbering is from the *XbaI* site as in Fig. 1. Sequence data are from refs 1, 2. Boxes enclose 10-bp sequences aligned in Fig. 5.

locus		5'					3'	match	reference										
mouse H	404	>	A	G	G	T	C	A	T	G	T	G	G	C	A	A	G	8	1,2
mouse H	533	<	T	A	C	C	C	A	G	G	T	G	T	G	T	T	8	1,2	
mouse H	353	<	A	G	T	C	A	A	G	A	T	G	G	C	C	G	A	7	1,2
mouse H	385	<	G	C	A	G	C	A	G	C	T	G	G	C	A	G	G	7	1,2
human H	89	>	A	C	A	G	C	A	G	G	T	G	G	C	A	G	G	8	12-14
human H	226	<	T	G	T	C	C	A	G	G	T	G	T	T	G	T	T	7	12-14
mouse k	77	<	C	T	G	C	C	A	G	A	T	G	G	C	C	T	C	9	11
human k	77	<	C	T	G	C	C	A	G	G	T	G	G	C	C	T	C	10	11
rabbit k	77	<	C	T	G	C	C	A	G	A	T	G	G	C	C	C	C	8	11
mouse k	129	>	C	A	G	G	C	A	G	G	T	G	G	C	C	C	A	9	10,11,15
human k	129	>	T	A	G	G	C	A	G	G	T	G	G	C	C	A	A	9	11
rabbit k	129	>	G	A	G	G	C	A	G	G	T	G	A	C	C	C	A	8	11
mouse k	180	>	G	T	C	C	C	A	T	G	T	G	G	T	T	A	C	7	10,11,15
rabbit k	199	>	C	G	G	C	C	A	G	G	T	G	C	A	A	G	G	8	11,37
Ig enhancers			G C C A G G T G G C																
frequency:			36 57 93 79 100 79 50 29																
			29 57 100 71 100 64 29																
viral enhancers			G T G G A A A G																
			T T T																
																		4	38
sv40 T	4073	<	T	T	G	C	C	A	G	G	T	G	G	G	T	T	A	9	39,40
pBR322	4281	<	A	C	G	T	C	A	G	G	T	G	G	C	A	C	T	9	41

Fig. 5 A consensus sequence for immunoglobulin intron enhancers. The position of guanines with altered DMS reactivity in the mouse heavy-chain enhancer in nuclei are indicated: ○, protected; ●, enhanced. Beneath the consensus sequence is the percentage of the matches found at that nucleotide position for the 14 enhancer segments aligned as shown. A weak similarity with a viral consensus³⁹ is also shown. The positions of the centres of the sequences are numbered in accordance with the references given. The symbols (<, >) indicate whether the sequence shown has been inverted relative to the original report or not, respectively. The column marked 'match' indicates the number of matches to the 10-bp consensus for the enhancer segment on that line.

This supports the hypothesis that tissue-specific genes make products that act on enhancers to turn on sets of genes during differentiation.

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Similarity between the vaccinia virus 19K early protein and epidermal growth factor

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An analysis of the 1,217-amino acid residue sequence of the precursor of mouse epidermal growth factor (mEGF)¹ revealed regions of considerable similarity with bovine factor X, a blood coagulation factor. Similarities of mEGF itself with factor X², pancreatic secretory trypsin inhibitor³ and, most strikingly, transforming growth factor I (TGF-I)^{4,5} have been observed. On the basis of the comparisons described here, it seems that the presumptive 140-residue 19K early protein (relative molecular mass (M_r) 19,000) of vaccinia virus⁶ from residues 40-91 shows an overall identity of 36% (19/53 residues) with both mEGF and urogastrone (human epidermal growth factor, hEGF); a single deletion is assumed for vaccinia virus 19K protein which allows the six Cys residues (positions 45-80) to be aligned with those of mEGF or hEGF. This protein is encoded in the 10.3-kilobase (kb) inverted terminal repeat⁶. Because it is an early protein with an EGF-like central portion, the 19K vaccinia virus protein may have an autocrine function and may be required for DNA synthesis.

The double-stranded DNA genome of vaccinia virus consists of ~182,000-186,000 nucleotide base pairs (bp)^{7,8} and can

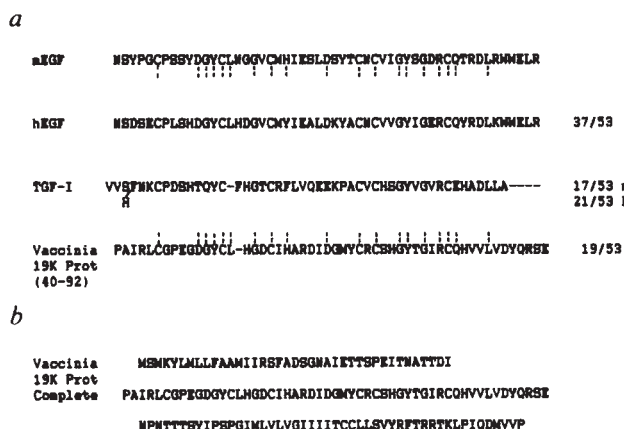


Fig. 1 *a*, Amino acid residue sequences for mEGF, urogastrone (hEGF), TGF-I and residues 40-92 of vaccinia virus 19K protein. The values on the right indicate the ratio of matches found with mEGF after alignment. Alignments were performed using the SEQA programme of Kanehisa *et al.*¹⁸. *b*, Complete sequence of the vaccinia virus 19K protein. Apart from the six Cys residues comparable with those in EGF, two additional ones are present at positions 118 and 119. Residues 6-15 and 106-123 are strongly hydrophobic.

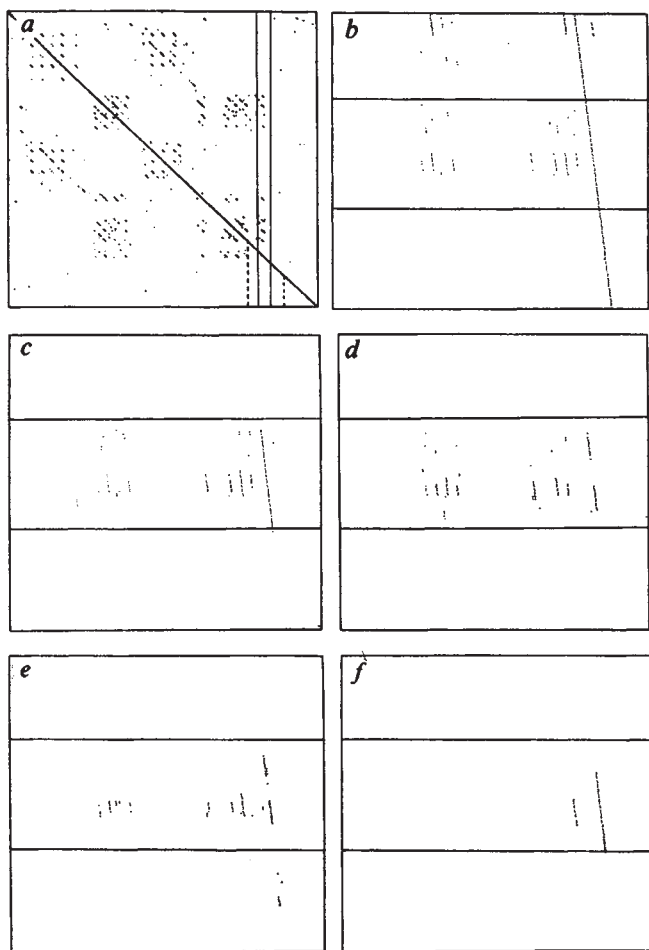


Fig. 2 Dot matrices showing positions of scored matches for pre-mEGF versus different amino acid residue sequences. The DIAGON¹⁵ program was used with the span length set to 11 and $P=0.0003$ (a-e) or span length 49, $P=0.00001$ (f), where P is the probability by chance alone of a scored match being found¹⁶. Dayhoff substitution scores^{2,15,16} were used. In each box the horizontal axis represents the 1,217 pre-mEGF sequence with the vertical axes indicated below. **a**, Pre-mEGF (residues 1-1,217); internal direct repeats are discernible and are not confined to the mEGF region. The vertical dashed lines delineate residues 937-1,076, that is, mEGF equals 937+40-1,076-47 and the vertical lines indicate the position of mEGF (residues 977-1,029). **b**, Pre-mEGF residues 937-1,076; horizontal lines delineate the mEGF region, with the mEGF-like sequences to the left of the 'main diagonal'. **c**, hEGF (urogastrone); the 53-residue sequence is padded with blanks above and below so that it is on a scale comparable with the 19K vaccinia virus protein. Matches are seen just below the lower line because of the persistence phenomenon caused by the scoring procedure¹⁶. The similarity of the substituted amino acids between mEGF and hEGF is evident. **d**, TGF-I; the 50 residue sequence is padded in a manner similar to **c**. **e**, Vaccinia virus 19K protein; some similarity exists beyond the EGF region. **f**, 19K vaccinia virus protein analysed at span length 49, $P=0.00001$.

encode >150 proteins⁹⁻¹¹. By virtue of the viral genome containing a complete transcriptional system, replication occurs in the cytoplasm; half of the genome consists of genes transcribed early in infection. One of these genes, contained in the 10.3-kb inverted terminal repeat (~6.91-7.39 kb from the end of the genome⁶), codes for the putative 140-residue polypeptide (15.5K) which is thought to be identical to the 19K protein observed on the polyacrylamide gels used to analyse the products of *in vitro* translation experiments¹².

The complementary DNA derived from the messenger RNA for mEGF has been sequenced^{13,14}; it is 4,749 bp long and codes for a primary translation product of 1,217 amino acid residues that may contain eight cryptic peptides, similar to mEGF. A

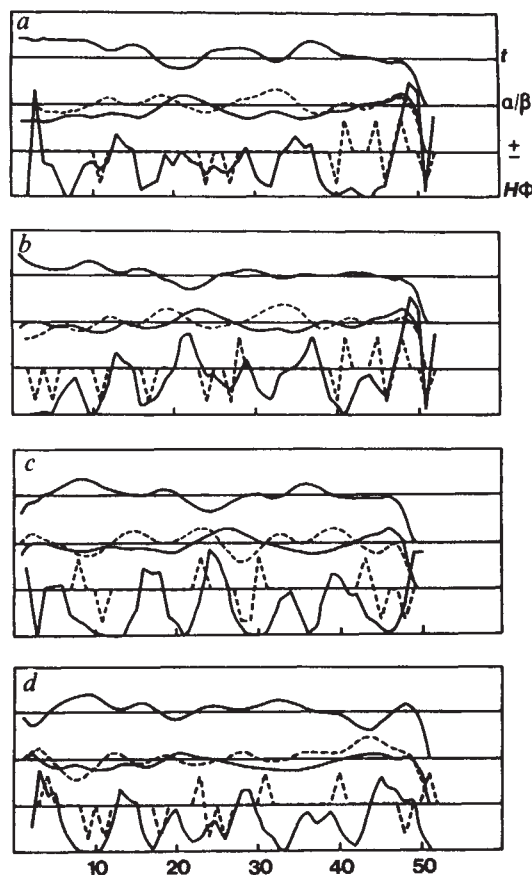


Fig. 3 Predictions of secondary structures for EGF and EGF-like peptides. Plots were obtained using software provided by Novotny and Auffray¹⁷. Determinations for α -helical, β -sheet and reverse-turn regions were based on Chou and Fasman parameters¹⁹ and the computations of hydrophobicity on the method of Rose and Roy²⁰. Overall, the plots are reasonably similar, particularly with respect to the hydrophobicity plots. **a**, mEGF; **b**, hEGF; **c**, rat TGF-I; **d**, vaccinia virus 19K protein, residues 40-92. The five curves from top to bottom of each group represent reverse-turn propensity (t), α -helix (—) and β -sheet (---) propensities, electric charge (+ above, - below the line), and hydrophobicity ($H\phi$). A single round of smoothing was used (seven-point window).

search of the National Biomedical Research Foundation's Protein Sequence Database (August 1984) revealed several sequences with pentapeptide or hexapeptide regions matched perfectly to regions contained in the 53 residues of either mEGF or hEGF. Only the 19K protein from vaccinia virus, however, contained the spacing of the Cys residues so characteristic of the peptides listed above. Figure 1 shows the sequence alignment for the EGFs, TGF-I and the 19K protein; Fig. 2 shows dot-matrix patterns for the peptides analysed against the pre-EGF sequence. These dot matrices were obtained using the DIAGON program developed by Staden¹⁵, which uses probability statistics developed by McLachlan¹⁶. Scoring was based on the Dayhoff amino acid substitution matrix². Figure 3 represents 'predictive' analyses for α -helical, β -sheet and reverse-turn regions as well as showing charge distributions and relative hydrophobicities ($H\phi$)¹⁷.

The analyses are consistent with the suggestion that the central region of the vaccinia virus 19K protein is quite similar to EGF. The flanking residues (1-39) give little indication of such similarity although there may be a suggestion of similarity (for some of the residues between 93 and 140) to amino acid residues on the -OH side of the EGF region in the pre-EGF peptide (Fig. 2f). It is clear also that both vaccinia virus 19K protein (residues 40-92) and TGF-I are more similar to EGF itself than to any of the EGF-like regions (Fig. 2). The propensity curves in Fig.

3 show similarities between both EGFs and TGF-I and vaccinia virus 19K protein. Note, however, that obvious differences exist between hEGF and mEGF as well as between TGF-I and vaccinia virus 19K protein and the two EGFs (Fig. 3a, c).

Although the function of the 19K protein is unknown, it is translated early during viral infection and its gene is present in the distal hypervariable region of the vaccinia genome⁶, consistent with its being co-opted from the host's genome. The analyses presented here suggest that the 19K protein has an

EGF-like function. Whether it is able to complex with EGF receptor sites (or sites similar to them) or whether the flanking sequences allow it to behave like an internalized and possibly degraded EGF-receptor complex remains to be elucidated.

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Cloning and expression in *Escherichia coli* of the gene for human tumour necrosis factor

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Tumour necrosis factor (TNF) was found originally in mouse serum after intravenous injection of bacterial endotoxin into mice primed with viable *Mycobacterium bovis*, strain Bacillus Calmette-Guerin (BCG)¹. TNF-containing serum from mice is cytotoxic or cytostatic to a number of mouse and human transformed cell lines, but less or not toxic to normal cells *in vitro*. It causes necrosis of transplantable tumours in mice. TNF also occurs in serum of rat¹, rabbit^{1,2} and guinea pig³. Rabbit TNF has been purified recently to give a single band on SDS-polyacrylamide gel electrophoresis (PAGE). The purified TNF had a relative molecular mass (M_r) 40,000 $\pm$ 5,000 measured by gel filtration, and 17,000 by SDS-PAGE. Its isoelectric point is 5.0 $\pm$ 0.3 (ref. 4). The necrotic activity *in vivo* and the cytotoxicity *in vitro* are produced by the same substance⁵. The gene encoding TNF has been identified in a human genomic DNA library using as a probe a cloned cDNA encoding a portion of rabbit TNF. The regions of this gene encoding an amino-acid sequence corresponding to mature TNF have been expressed in *Escherichia coli* and the product of this expression isolated in pure form and shown to produce necrosis of murine tumours *in vivo*.

Recently, rabbit complementary DNA (cDNA) prepared from the messenger RNA (mRNA) extracted from rabbit alveolar cells has been cloned in *E. coli* (H.I. *et al.*, manuscript in preparation). Expression of the cloned cDNA modified to express the serum (mature) form of rabbit TNF in *E. coli* gave a product which was cytotoxic to a transformed cell line and caused a necrotic response to an experimental tumour (H.I. *et al.*, manuscript in preparation). There is less information about human TNF⁶⁻⁸ which has not yet been purified.

A human genomic library prepared by inserting large *EcoRI*-methylated fragments from human DNA partially digested with *HaeIII* and *AluI* into Charon 4A λ phage⁹ using *EcoRI* linkers was obtained from T. Maniatis¹⁰. A rabbit TNF cDNA probe (pB2-7, H.I. *et al.*, manuscript in preparation) labelled by nick translation^{11,12} with [α -³²P]dTCP enabled identification of nine positive clones, designated HG-1 to HG-9, in this library. The phage DNA from each of these clones was isolated using the

miniprep method^{13,14}. Each of the nine clones was examined by restriction enzyme analysis and found to share a common 2.9-kilobase (kb) *EcoRI* fragment complementary to the pB2-7 probe. The phage DNA was isolated from one of these clones, designated HG-3, and the 2.9-kb *EcoRI* fragment was isolated and ligated into the *EcoRI* site of pUC13 (ref. 15) to obtain a plasmid, designated pHGE.

The insert from pHGE was sequenced using the method of Maxam and Gilbert¹⁶ by the strategy shown in Fig. 1a. As the pHGE insert did not contain the polyadenylation site, a *HindIII* fragment overlapping the 3' end of the pHGE insert was isolated from HG-3 DNA and inserted into the *HindIII* site of pUC13 to yield a plasmid designated pHGH2 (Fig. 1b). Sequencing of the *HindIII* insert of this plasmid identified the presumed polyadenylation site in this segment. The total sequence of the human genomic TNF gene with exons and introns identified is presented in Fig. 1c. The amino-acid sequence deduced from the DNA encoding the recombinant mature TNF indicates that the natural human TNF does not have N-glycosylation sites¹⁷ and has only one possible S-S bridge.

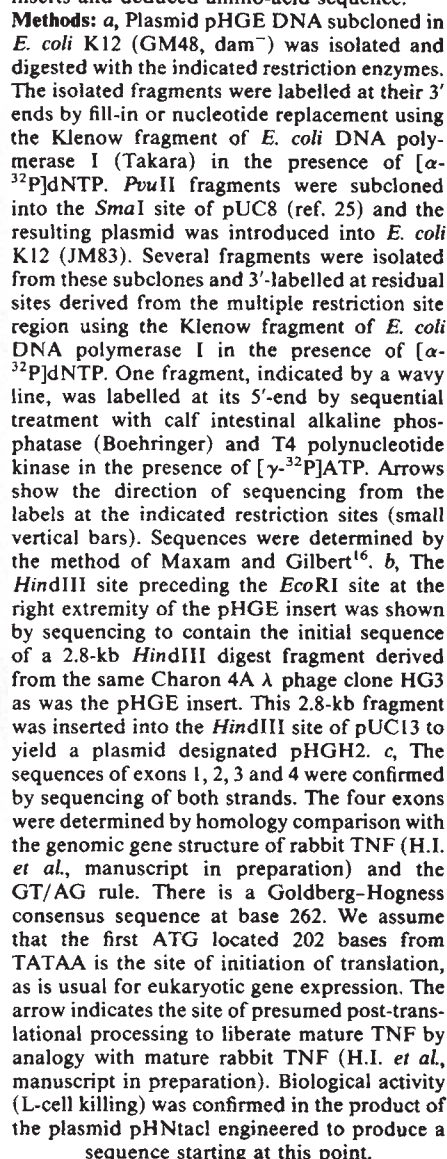
By analogy with the genomic gene structure of rabbit TNF, we expect that the protein expressed initially from the mRNA derived from this gene would be processed to yield a product with the N-terminal sequence Ser-Ser-Ser (exon 3, Fig. 1c). An expression vector encoding such a mature TNF sequence was constructed by combining a major portion of exon 4 with four synthetic DNA fragments under the control of the *tac* promoter¹⁸ as shown in Fig. 2. This approach avoided the need for a source of human mRNA.

The purification of recombinant human TNF produced in *E. coli* (Fig. 3) was monitored by mouse L-cell killing activity. The M_r estimated by gel filtration (Fig. 3a) is 45,000. SDS-PAGE

Table 1 Tumour necrotic activity of human TNF

Dose (U in 0.05 ml)	Necrotic response				Cured ratio
	+++	++	+	-	
	(No. of mice)				
5,000	3	1			2/4
500			4		0/4
50			1	3	0/4
Control				4	0/4

Colon 26 adenocarcinoma (0.1 ml of 2% tumour brei) was transplanted intradermally into BALB/c mice on day 0. The diameter of the tumour mass was ~10 mm on day 9, when purified recombinant human TNF dissolved in saline containing gelatin (1 mg ml⁻¹) was injected intratumorally. The control group was treated with saline containing gelatin (1 mg ml⁻¹). Necrotic responses were scored according to the method of Carswell *et al.*¹ and tumours of cured mice were completely regressed.



Although the human TNF produced in *E. coli* cannot be compared directly with natural human TNF (which has yet to be isolated) we believe that the purified human TNF produced

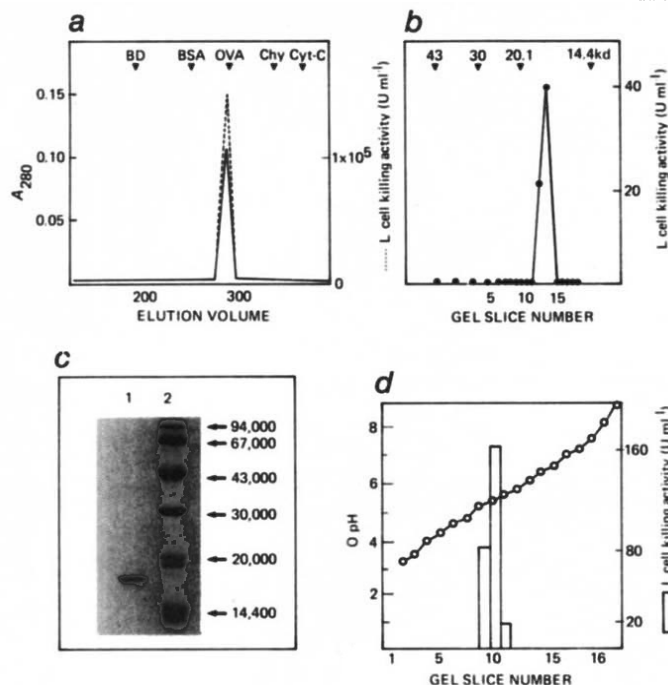


Fig. 2 Construction of plasmids. *a*, Plasmid pHGE; map derived from sequencing information (described in Fig. 1). *b*, Plasmid pHNtac4 encoding the synthesis of human mature TNF in *E. coli*. **Methods:** pHGE DNA was digested completely with *Xho*I and *Sal*I. The desired 750-bp fragment was isolated and digested completely with *Hinc*II and the resulting 250-bp and 450-bp fragments isolated. The 250-bp fragment was digested partially with *Dde*I and a 206-bp fragment was recovered. The four oligodeoxynucleotides shown above were synthesized by the modified triester method²⁶. A mixture of these oligodeoxynucleotides was phosphorylated using ATP and T4 polynucleotide kinase, then the 206-bp fragment was added and the components ligated with T4 DNA ligase. The product was digested with *Bam*HI and *Hinc*II and a 260-bp oligodeoxynucleotide isolated from the digest. Plasmid pGtac1110 contains the *tac* promoter from ptacl1 (ref. 18) inserted in clockwise orientation in the *Hind*III site of pBR327, followed by the sequence ATCTAT interposed between the initial G of the *Eco*RI site and the start codon of a coding sequence for γ -interferon (C.W.T., Y. Takahashi and J. Rossi, unpublished results). The sequence of pBR327 resumes at the *Sal*I site. pGtac1110 was digested with *Bgl*II and *Sal*I and the large vector fragment recovered. The 450-bp and the 260-bp fragments were ligated into this vector fragment; transformation of *E. coli* K-12 (D-1210) gave 50 ampicillin-resistant colonies. Plasmid DNA was prepared from 12 of these transformants and digested with *Hind*III. Two of these plasmids contained the desired 852-bp fragment; DNA sequence analysis verified that these plasmids had the desired sequence of promoter, synthetic DNA insert and genomic DNA. One plasmid was designated pHNtac4.

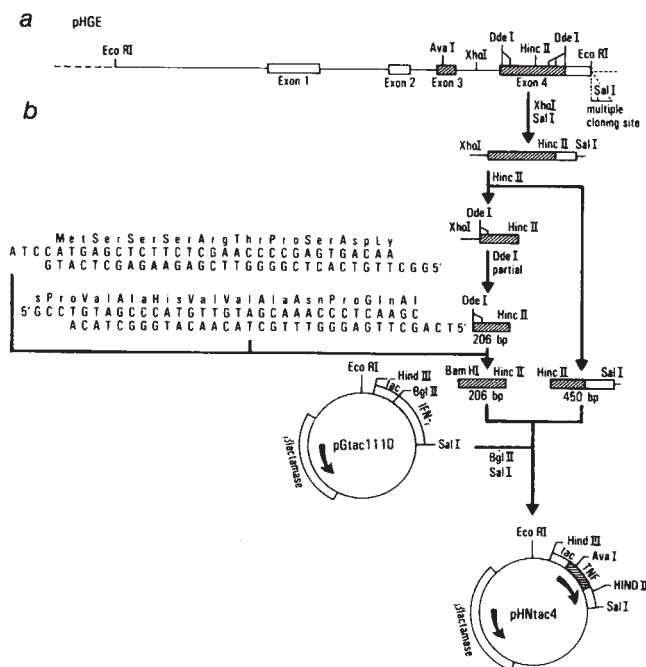


Fig. 3 Purification and properties of mature human recombinant TNF. *a*, Filtration on Sephacryl S-200; *b*, *c*, SDS-PAGE; *d*, isoelectric focusing.

Methods: Human TNF produced in *E. coli* was purified to homogeneity. The activity of human TNF during purification was monitored by mouse L-cell killing activity by a modification of the method of Williamson *et al.*⁸ using L-M cells (American Type Culture Collection, CCL 1.2). The number of surviving cells was determined by the photometric method of Ruff and Gifford²⁷. Cells from a culture of *E. coli* K12 (D1210)/pHNtac4 in Luria broth containing 50 μ g ml⁻¹ ampicillin were collected by centrifugation and passed 10 times through a Manton-Gaulin press (Gaulin Corp.). The supernatant cell lysate had a specific activity of 32,000 U mg⁻¹ of protein. The cell lysate was loaded onto a DEAE-Sephacrose CL-6B (Pharmacia) column equilibrated with 20 mM Tris-HCl, pH 8.0 and eluted at pH 8.0 with 20 mM Tris-HCl containing 100 mM NaCl. Fractions with L-cell killing activity were pooled and further concentrated in a polysulphone hollow fibre (Asahi) with an *M_r* exclusion limit of 6,000. The concentrate was heated at 60 °C for 30 min with gentle stirring followed by filtration through a 0.2 μ m membrane (Flow Laboratory, U.S.A.). The sample was loaded onto another DEAE-Sephacrose CL-6B column (2.6 $\times$ 11 cm) equilibrated with 20 mM Tris-HCl, pH 8.0, 150 mM NaCl. The active material was eluted with 20 mM Tris-HCl, 150 mM NaCl, pH 8 and the active fractions pooled and loaded onto a Sephacryl S-200 column (Pharmacia) in 5 mM sodium phosphate, 150 mM NaCl, pH 7.4. The active fractions corresponded to *M_r* ~45,000 with 41% recovery of TNF activity. This material of specific activity 1.4 $\times$ 10⁶ U mg⁻¹ of protein, was used for characterization studies. *a*, The TNF sample was applied to Sephacryl S-200 column. *M_r* was determined by previously calibrating the column with known standard samples. (Blue dextran 2000, bovine serum albumin (*M_r* 67,000), ovalbumin (*M_r* 45,000), chymotrypsinogen A (*M_r* 25,000), cytochrome *c* (*M_r* 12,500). *b*, Molecular size and homogeneity of protein preparations were determined by electrophoresis on 15% polyacrylamide gel with SDS²⁸; proteins of known *M_r* served as internal markers. After electrophoresis the gel was washed with distilled water for 1 h and sliced in 2–5 mm pieces. The slices were extracted overnight with 5 mM sodium phosphate buffer, pH 7.4, containing 100 μ g ml⁻¹ of HCO-60 (polyoxyethylene hydrogenated castor oil derivative, Nikko Chemicals). *c*, As above, except that the gel was stained with Coomassie brilliant blue R-250. Lane 1; TNF from preparative PAGE; lane 2, *M_r* markers: phosphorylase B (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,000), α -lactalbumin (14,400). *d*, Isoelectric focusing of a cell lysate was performed as described in ref. 29. After focusing the gel was sliced, extracted overnight with 5 mM sodium phosphate buffer, pH 7.4, containing 100 μ g ml⁻¹ of HCO-60 at 4 °C.

in *E. coli* has almost the same or an identical structure to the natural human TNF. If so, it seems probable that the recombinant material thus produced may find application in therapy of cancer and possibly also of malaria^{21–24}.

We thank Dr Bonnie Mills for assays of L-cell killing activity and Professor T. Maniatis for providing the Charon 4A λ phage human genomic library. The research reported here was performed while T.S. and H.Y. were Visiting Research Fellows at the Beckman Research Institute of the City of Hope. Present address is Technical Research Laboratory, Asahi Chemical Industry Co., Ltd., 2-1 Samejima, Fuji-Shi, Shizuoka, Japan.

Note added in proof: While this paper was in press, Pennica *et al.*³⁰ reported similar results from expression of cloned cDNA. The deduced amino-acid sequences are in complete agreement except for the assignment of the point of cleavage for mature TNF. The overlapping nucleotide sequences differ in four non-coding positions: 440 (T), 2,462 (G), 2,553 (A) and 2,997 (A) (Fig. 1c) against 125 (C), 1,053 (T), 1,144 (G) and 1,588 (G) (Fig. 1b in ref. 30).

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Isolation and characterization of genomic and cDNA clones of human erythropoietin

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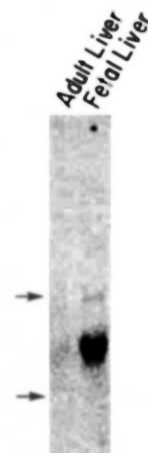
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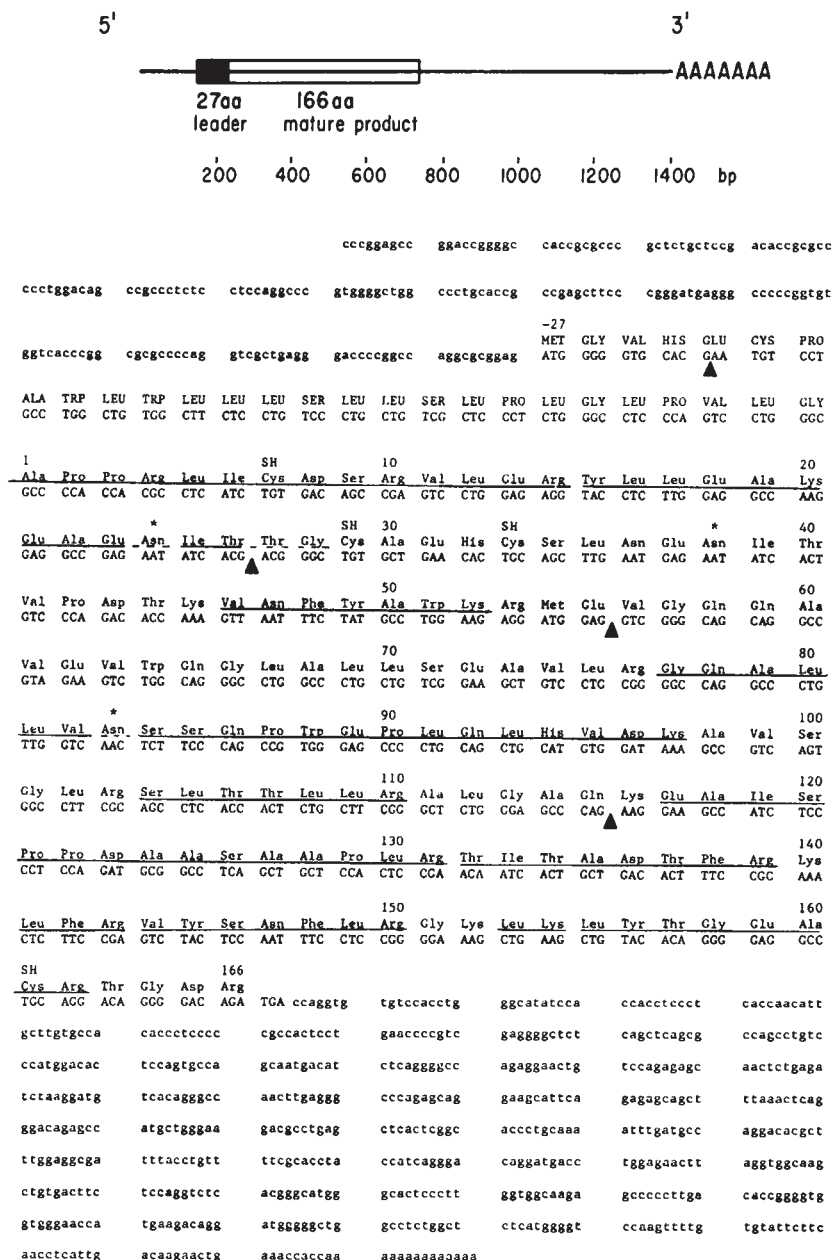
The glycoprotein hormone erythropoietin regulates the level of oxygen in the blood by modulating the number of circulating erythrocytes, and is produced in the kidney¹⁻⁴ or liver^{5,6} of adult and the liver^{7,8} of fetal or neonatal mammals. Neither the precise cell types that produce erythropoietin nor the mechanisms by which the same or different cells measure the circulating oxygen concentration and consequently regulate erythropoietin production (for review see ref. 9) are known. Cells responsive to erythropoietin have been identified in the adult bone marrow¹⁰, fetal liver¹¹ or adult spleen¹². In cultures of erythropoietic progenitors, erythropoietin stimulates proliferation and differentiation to more mature red blood cells. Detailed molecular studies have been hampered, however, by the impurity and heterogeneity of target cell populations and the difficulty of obtaining significant quantities of the purified hormone. Highly purified erythropoietin may be useful in the treatment of various forms of anaemia, particularly in chronic renal failure¹³⁻¹⁵. Here we describe the cloning of the human erythropoietin gene and the expression of an erythropoietin cDNA clone in a transient mammalian expression system to yield a secreted product with biological activity.

Fig. 1 Northern analysis of human fetal liver mRNA (5 µg) and adult liver mRNA (5 µg) were electrophoresed in a 0.8% agarose/formaldehyde gel and transferred to nitrocellulose as described previously⁴¹. An erythropoietin-specific single-stranded probe was prepared from an M13 template containing the 87-bp exon of the human erythropoietin gene; the primer was a 20 mer derived from the same tryptic fragment as the original 17 mer probe. The ³²P-labelled probe was prepared as described previously⁴² except that after digestion with *Sma*I, the small fragment was purified from the M13 template by chromatography on a Sepharose CL4b column in 0.1 M NaOH/0.2 M NaCl. The filter was hybridized to ~5 × 10⁶ c.p.m. of this probe for 12 h at 68 °C, washed in 2 × SSC at 68 °C and exposed for 6 days with an intensifying screen. Marker mRNAs of ~2,200 and 1,000 nucleotides (indicated by arrows) were run in an adjacent lane.



Methods. Erythropoietin was purified as described previously²⁷ except that the phenol treatment was eliminated and replaced by heat treatment at 80 °C for 5 min to inactivate neuraminidase and the final step in the purification was fractionation on a C-4 Vydac reverse-phase HPLC column (Separations Group) using a 0-95% acetonitrile gradient in 0.1% trifluoroacetic acid (TFA) over 100 min. The position of erythropoietin in the gradient was determined by gel electrophoresis and N-terminal amino-acid sequence analysis¹⁶ of the major peaks and comparing sequences obtained with those previously reported for erythropoietin²¹⁻²³. Using this approach, erythropoietin was shown to elute at ~53% acetonitrile and represented 40% of the total eluted protein. Fractions containing erythropoietin were evaporated to ~100 µl, adjusted to pH 7 with 1 M ammonium bicarbonate and digested to completion with TPCK-treated trypsin (Worthington) (2% w/w enzyme/substrate) for 18 h at 37 °C. The tryptic digest was then subjected to reverse-phase HPLC using the conditions described above and the absorbance at both 280 and 214 nm monitored. Well-separated peaks were evaporated to near dryness and subjected directly to N-terminal sequence analysis¹⁶ using an Applied Biosystems Model 470A gas phase sequencer. The sequences obtained are underlined in Fig. 2. Two of these tryptic fragments were chosen for synthesis of oligonucleotide probes. From the sequence Val-Asn-Phe-Tyr-Ala-Trp-Lys a 17 mer of 32-fold degeneracy (5'd(TTCCANGCG⁺TAG⁺AAG⁺TT); pool I) and a partially overlapping 18 mer of 128-fold degeneracy (5'd(CCANGCG⁺TAG⁺AAG⁺TTNAC); pool II) were prepared on an Applied Biosystems Model 380A DNA synthesizer. From the sequence Val-Tyr-Ser-Asn-Phe-Leu-Arg. two pools of 14 mers, each 48-fold degenerate (5'd(TAC⁺TA⁺G⁺CT⁺NAAT⁺TTT⁺CT); pool III) and 5'd(TAC⁺TA⁺G⁺CT⁺NAAT⁺TTT⁺TT); pool IV), which differ at the first position of the leucine codon, were prepared. The oligonucleotides were labelled at the 5' end using polynucleotide kinase (New England Biolabs) and [γ -³²P]ATP (NEN). The specific activity of the oligonucleotides varied between 1,000 and 3,000 Ci mmol⁻¹ oligonucleotide. A human genomic DNA library in bacteriophage λ ⁴³ was screened using a modification of the *in situ* amplification procedure described originally by Woo *et al.*⁴⁴ and using tetramethylammonium chloride as the hybridization salt (see also refs 45-47; K.J. *et al.*, in preparation). Two independent phage (designated λ HEPO1 and λ HEPO2) hybridized to all three probes. DNA from λ HEPO1 was digested to completion with *Sau*3A and subcloned into M13 for DNA sequence analysis using the dideoxy chain termination method⁴⁷. Analysis of this DNA sequence revealed an open reading frame which precisely codes for the tryptic fragment used to deduce pool I. This open reading frame was contained in an 87-bp exon, bounded by potential splice acceptor and donor sites. Confirmation that λ HEPO1 and λ HEPO2 contain portions of the erythropoietin was obtained by identification, through further DNA sequencing of additional exons encoding amino-acid sequences corresponding to previously determined sequences of tryptic fragments of purified erythropoietin (see Figs 2, 3).

Fig. 2 Nucleotide and amino-acid sequence of an erythropoietin fetal liver cDNA. A 95-nucleotide probe identical to that described in Fig. 1 was prepared and used to screen a fetal liver cDNA library in the vector λ Ch21A²⁰ using standard plaque screening⁴⁸ procedures. Three independent positive clones (designated λ HEPOFL6 (1,350 bp), λ HEPOFL8 (700 bp) and λ HEPOFL12 (1,400 bp)) were isolated following screening of 1×10^6 plaques. The entire insert of λ HEPOFL13 was sequenced following subcloning into M13. The 5'- and 3'-untranslated sequences are in lower case letters, the coding region in upper case letters. Small filled triangles indicate positions of introns as determined from sequencing of the erythropoietin gene (Fig. 3). The deduced amino-acid sequence is given above the nucleotide sequence and is numbered beginning with 1 for the first amino acid of the mature protein. The putative leader peptide is indicated by capital letters for the amino-acid designations. Cysteine residues in the mature protein are indicated additionally by SH and potential N-linked glycosylation sites by an asterisk. The underlined amino acids indicate those residues identified by N-terminal protein sequencing or by sequencing tryptic fragments of erythropoietin as described in Fig. 1. Partial underlining indicates residues in the amino-acid sequence of certain tryptic fragments which could not be determined unambiguously. Partial DNA sequence analysis indicated that λ HEPOFL8 contained an additional 39 nucleotides of the 5'-untranslated sequence (see Fig. 3) and ended at the Arg codon at amino-acid position 162, but was otherwise identical to λ HEPOFL13 in the residues sequenced. Complete sequence analysis of λ HEPOFL6 indicated that it was identical to λ HEPOFL13 except that the 5'-untranslated sequence and first 13 nucleotides of the coding region were absent and replaced by the 3' 107 nucleotides of the intron between exons I and II (see Fig. 3). Thus, the λ HEPOFL6 cDNA clone seems to be derived from a partially spliced mRNA that processed out correctly all intervening sequences except for the one between exons I and II.

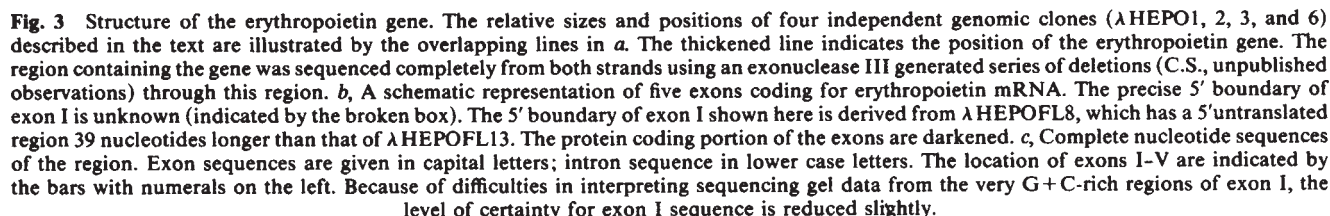


Approximately 10 μ g of human erythropoietin was purified from the urine of patients with aplastic anaemia and digested to completion with trypsin. The tryptic fragments were then purified by reverse-phase HPLC and subjected to microsequence analysis (ref. 16; see Fig. 1). We prepared highly degenerate synthetic oligonucleotides based on the amino-acid sequences and used these oligonucleotides to isolate the erythropoietin gene from a bacteriophage λ library of human genomic DNA (see Fig. 1).

The erythropoietin genomic clones were then used to determine whether human fetal liver is a potential source of messenger RNA for complementary DNA cloning, because erythropoietin is released from mouse¹⁷, sheep¹⁸ and human¹⁹ fetal liver. Human fetal (20-week-old) and adult liver mRNAs were analysed by Northern blotting using as a probe a 95-nucleotide single-stranded fragment containing the 87-base pair (bp) exon described in Fig. 1. A strong signal was detected in fetal liver mRNA corresponding to an mRNA ~1,600 nucleotides in length (Fig. 1). An mRNA of identical size was detected weakly in adult liver mRNA and transcripts of ~2,000 nucleotides were detected weakly in both fetal and adult mRNA. The same probe was then used to isolate cDNA clones from a bacteriophage λ cDNA library constructed from the fetal liver mRNA²⁰.

The complete nucleotide and deduced amino-acid sequence for the largest of these clones (designated λ HEPOFL13) is shown in Fig. 2. The erythropoietin coding information is contained in 579 nucleotides in the 5' half of the cDNA and encodes a hydrophobic 27-amino-acid leader peptide followed by the 166-amino-acid mature protein. The identification of the N-terminus of the mature protein is based on the N-terminal sequence of the protein secreted in the urine of patients with aplastic anaemia as determined originally by Goldwasser^{21,22} and later confirmed (Fig. 1 and ref. 23). The amino acids underlined in Fig. 2 indicate the protein sequences obtained (see Fig. 1 legend) either from the N-terminus of intact erythropoietin or from purified tryptic fragments. The deduced amino-acid sequence agrees precisely with the protein sequence data, confirming that the isolated cDNA encodes human erythropoietin.

To demonstrate that biologically active erythropoietin could be expressed from the cloned cDNA, we performed transient expression experiments in COS cells²⁴. The vector (p91023B) contains the adenovirus major late promoter, a simian virus 40 (SV40) polyadenylation sequence, an SV40 enhancer and origin of replication and the adenovirus virus-associated (VA) gene^{25,26}. Erythropoietin cDNA was inserted into the p91023B vector downstream of the adenovirus major late promoter (Fig.



Thus, the protein originally purified by Miyake *et al.*²⁷ and containing the N-terminus Ala-Pro-Pro-Arg... is erythropoietin (refs 21, 23; Fig. 2). Western blotting (using a polyclonal anti-erythropoietin antibody) indicates that erythropoietin produced in COS cells has a mobility on SDS-polyacrylamide gels identical to that of the native hormone prepared from human urine (data not shown).

As well as the clones described above (λ HEPO1 and λ HEPO2), two other genomic clones (λ HEPO3 and λ HEPO6) were isolated in subsequent screens of the human genomic library (Fig. 3a). Hybridization analysis of the cloned DNAs with oligonucleotide probes and with probes prepared from the erythropoietin cDNA clones positioned the erythropoietin gene in the 3.3-kilobase (kb) region in Fig. 3a. Complete sequence analysis of this region and comparison with the cDNA clones gave the map of intron and exon structure of the erythropoietin gene (Fig. 3b, c); the erythropoietin mRNA is encoded by at

Table 1 Assay for detection of erythropoietin activity

Assay method	Activity
<i>In vitro</i> CFU-E	$2.0 \pm 0.5 \text{ U ml}^{-1}$
<i>In vitro</i> ^3H -thymidine	$3.1 \pm 1.8 \text{ U ml}^{-1}$
<i>In vivo</i> exhypoxic mouse	1 U ml^{-1}
<i>In vivo</i> , starved rat	2.4 U ml^{-1}

The cDNA insert from λ HEOPOFL13 was inserted into the vector p91023B (ref. 25) described in the text. Purified DNA (8 μg) was then used to transfect 5×10^6 M6 COS cells³⁷ using the DEAE-dextran method²⁵; 12 h after transfection the cells were washed and exposed to media containing 10% fetal calf serum for 24 h. Cells were then changed to 4 ml serum-free media and collected 48 h later. *In vitro* biologically active erythropoietin was measured using either a colony-forming assay with mouse fetal liver cells as a source of erythroid colony-forming units (CFU-E)³⁸ or a ^3H -thymidine uptake assay using spleen cells from phenylhydrazine-injected mice¹². Activities are expressed in units ml^{-1} , using a commercial, quantified erythropoietin (Toyobo, Inc.) as a standard. The sensitivities of the assays are $\sim 25 \text{ mU ml}^{-1}$. *In vivo* biologically active erythropoietin was measured using either the hypoxic mouse³⁹ or the starved rat⁴⁰ method. The sensitivities of these assays are $\sim 100 \text{ mU ml}^{-1}$. No activity was detected in either assay from mock-conditioned media. In subsequent experiments with the same vector, expression levels as high as $25 \pm 3 \text{ U ml}^{-1}$ (^3H -thymidine assay method) have been observed.

least five exons. Exons II, III, IV and parts of I and V contain the protein coding information, whereas the rest of exons I and V encode the 5'- and 3'-untranslated sequences, respectively. Exon I is 80% G+C and is surrounded by sequences equally G+C-rich. The CpG dinucleotide frequency in this region ($\sim 10\%$) is not significantly under-represented as it is in the remainder of the gene ($\sim 2\%$) and thus suggests a region of high methylation. The location of the actual cap site and the promoter region are not yet known.

The 166-amino-acid sequence deduced from the cDNA clones agrees precisely with our 102 amino acids of partial sequence of human urinary erythropoietin, including 25 residues at the N-terminus and 77 residues in 9 internal tryptic fragments. The sequence differs at four positions from the N-terminal sequences previously published²¹⁻²³, probably because of errors in interpretation or assignments in the original sequencing. The extent of identity between native human erythropoietin and the gene isolated here and the fact that we can detect only a single gene by genomic blotting with erythropoietin cDNA probes (data not shown) implies that the gene we have isolated is not a pseudogene or a closely related variant of the erythropoietin gene. If a second gene exists, it must be highly homologous over many kilobases to the gene described here.

We have assigned the N-terminus of the mature protein based on the N-terminus of the protein released into urine of individuals with aplastic anaemia, consistent with the hypothesis that the preceding 27 highly hydrophobic amino acids constitute a secretory leader peptide. One or more of the amino acids preceding the presumed mature terminus may be normally secreted with the remaining protein as a pro-form of erythropoietin, later processed to the native N-terminus. Amino-acid sequence analysis of tryptic fragments of urinary erythropoietin has not yet identified the fragment containing the C-terminal four amino acids (Thr-Gly-Asp-Arg; see Fig. 2). Thus, processing of erythropoietin may occur at the C-terminus and some or all of the final four amino acids encoded in the cDNA may be removed in this way. C-terminal sequencing of native erythropoietin or identification of the fragment will be necessary to answer this question.

There are four cysteines in the 166 amino acids of mature erythropoietin. Based on the sensitivity of the biological activity of erythropoietin to reducing agents (ref. 28 and T. Shimizu, personal communication), at least two of these residues must be involved in a disulphide bond.

In the mature protein there are three predicted sites of N-linked glycosylation (residues 24, 38 and 83) based on the consensus glycosylation site Asn-X-Ser/Thr²⁹. Amino-acid

sequence analysis suggests that the asparagines at residues 24 and 83 are glycosylated (data not shown) (residue 38 has not been examined). Native erythropoietin is highly glycosylated, displaying a complex, probably poly-antennary sugar structure³⁰. The relative molecular mass (M_r) of the protein backbone deduced from the primary sequence is 18,398. As the reported M_r s for native erythropoietin determined by SDS gel electrophoresis are in the range 34,000–39,000 (refs 27, 31), nearly one-half of the apparent M_r of erythropoietin must be contributed by the sugar side chains. Whether any of the glycosylation is the result of O-linked glycosylation is unknown. The terminal sialic acid residue(s) of native erythropoietin is required for full *in vivo* biological activity but is not necessary for *in vitro* activity³². This effect may result from enhanced clearance of asialylated erythropoietin from the circulation by the liver³³. The biological activity of a completely unglycosylated erythropoietin may now be assessable using a recombinant system.

Lee-Huang³⁴ recently reported the isolation of an erythropoietin cDNA clone from mRNA of a human kidney carcinoma. As no sequence information was provided, we are unable to compare the erythropoietin clones described here with the cDNA clone of Lee-Huang³⁴. Fyhrquist *et al.*³⁵ have suggested that renin substrate (angiotensinogen) may be the erythropoietin precursor. Our results argue against a large precursor and comparison of the human erythropoietin amino-acid sequence with the rat angiotensinogen protein sequence³⁶ reveals no regions of homology and further argues against any relationship between the two polypeptides. Finally, extensive comparison of the erythropoietin amino-acid and cDNA sequence with sequences contained in both the National Biomedical Research Foundation and Genbank data bases has revealed no significant homology with any published sequence.

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Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter

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Although promoter regions for many plant nuclear genes have been sequenced, identification of the active promoter sequence has been carried out only for the octopine synthase promoter¹. That analysis was of callus tissue and made use of an enzyme assay. We have analysed the effects of 5' deletions in a plant viral promoter in tobacco callus as well as in regenerated plants, including different plant tissues. We assayed the RNA transcription product which allows a more direct assessment of deletion effects. The cauliflower mosaic virus (CaMV) 35S promoter provides a model plant nuclear promoter system, as its double-strand DNA genome is transcribed by host nuclear RNA polymerase II from a CaMV minichromosome². Sequences extending to -46 were sufficient for accurate transcription initiation whereas the region between -46 and -105 increased greatly the level of transcription. The 35S promoter showed no tissue-specificity of expression.

The 35S promoter region was isolated as a *Bgl*III fragment extending from -941 to +208 with respect to the transcription start site mapped for the 35S RNA found in CaMV-infected turnip leaves³. The polyadenylation site for the 19S and 35S CaMV transcripts located at +180 (ref. 3) was deleted, as described in Fig. 1 legend, to eliminate any possible processing signals in the promoter fragment. A 3' deleted promoter fragment extending to +9 was deleted at its 5' end (see Fig. 1) and fragments extending to -343, -168, -105 and -46 were chosen for analysis.

An abbreviated human growth hormone gene (*hgh*)⁴ was added as a test gene downstream to the 35S promoter deletion fragments. Information on plant cell recognition of animal gene splice and 3' polyadenylation signals obtained from analysis of *hgh* RNA transcribed in transformed plant cells will be presented elsewhere (A. Hunt, N. Chu, J.T.O., F.N. and N.-H.C., in preparation). The 35S promoter-*hgh* chimaeric gene was inserted in the pMON178 tumour-inducing (Ti)-plasmid vector, a derivative of pMON120 (ref. 5). Included in this vector is the nopaline synthase (NOS) promoter placed 5' to the neomycin phosphotransferase-II (*npt-II*) coding region (NOS promoter-*npt-II* gene), which is co-transferred with the 35S promoter-*hgh* gene into the tobacco genome and provides an internal standard for comparison of the activities from different 35S promoter deletion fragments.

Following tri-parental matings^{5,6}, *Agrobacterium tumefaciens* containing both chimaeric genes was used to infect SR1 *Nicotiana tabacum* cells by wounding⁵ and co-cultivation^{5,7}.

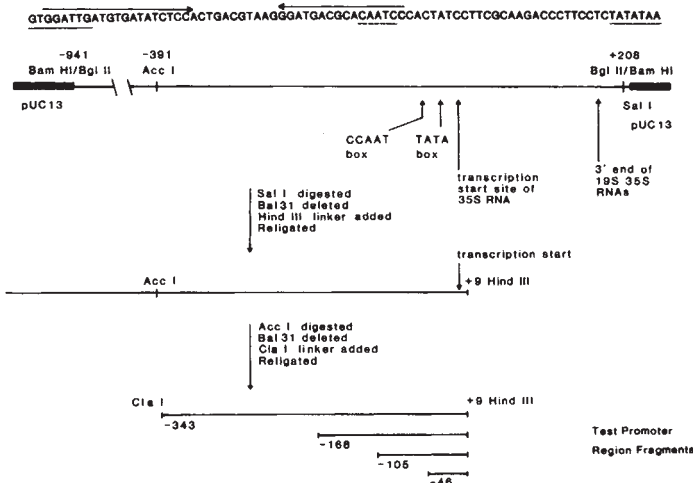


Fig. 1 Construction of 35S promoter region fragments. A 1.15-kb *Bgl*III fragment was subcloned from pCS101, a clone containing the entire *Cabb-S* CaMV genome³, into the *Bam*HI site of pUC13. The resulting plasmid was linearized at the *Sal*I site in the pUC13 polylinker next to the 3' end of the promoter fragment, digested with *Bal*31 exonuclease¹¹, ligated to *Hind*III linkers and recircularized. Clones were analysed for the extent of 3' deletion by polyacrylamide gel sizing of the *Acc*I/*Hind*III fragments and finally by dideoxy sequencing¹² of subclones in pUC using the universal primer. The plasmid containing a 3' deletion fragment with the *Hind*III linker at +9 was linearized with *Acc*I (site at -391), digested with *Bal*31 exonuclease, ligated to *Cla*I linkers and recircularized. Clones were analysed for the extent of 5' deletion by polyacrylamide gel sizing of the *Cla*I/*Hind*III fragment, followed by dideoxy sequencing of subclones in pUC using either the universal primer or primer generation by exonuclease III digestion¹³. Above is the sequence of the -105 to -25 region of the 35S promoter¹⁴ with TATA-box, CAAT-box, inverted repeat and core enhancer sequence regions marked.

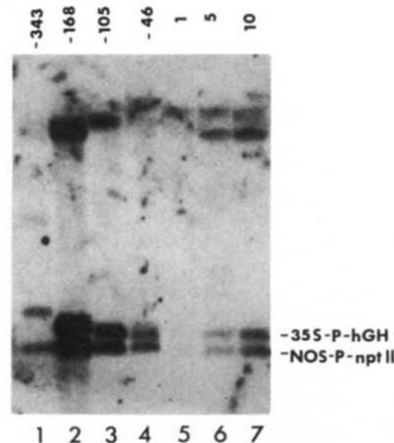


Fig. 2 Southern blot analysis of DNA from transformed tobacco calli. DNA was prepared, digested with *Eco*RI, electrophoresed on a 0.7% agarose gel and blotted onto a nitrocellulose filter¹⁵. A plasmid constructed to serve as the hybridization probe contains a *Bam*HI/*Sma*I *hgh* gene fragment and a *Bam*HI/*Bgl*III *npt-II* gene fragment cloned into pUC12 (GH-Neo24). The plasmid was nick translated¹⁶ and hybridized to the Southern blot by the method of Thomashow *et al.*¹⁷. The following samples contain 15 µg of calli DNA transformed with: lane 1, -343 35S promoter-*hgh*; lane 2, -168 35S promoter-*hgh*; lane 3, -105, 35S promoter-*hgh*; lane 4, -46 35S promoter-*hgh*. Reconstructions of the NOS promoter-*npt-II* gene and 35S promoter-*hgh* gene copy numbers contain 15 µg of control untransformed plant DNA mixed with different amounts of the pMON178 plasmid containing the -105 35S promoter-*hgh* gene: lane 5, 17 pg = 1 copy; lane 6, 85 pg = 5 copies; lane 7, 170 pg = 10 copies. The bands near the top of the filter in lanes 1-4 result from hybridization of the pBR322 sequences in the GH-Neo24 probe plasmid to pBR322 sequences in the integrated pMON178 Ti vector. In lanes 5-7 the upper bands are derived from other regions of the pMON178 plasmid.

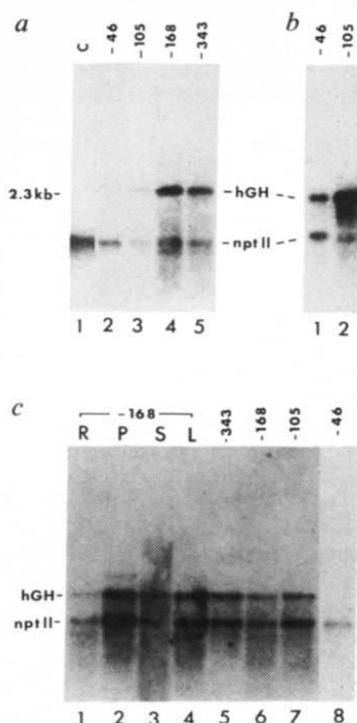


Fig. 3 Northern blot analysis of transformed tobacco calli transcripts. RNA was prepared from calli using guanidinium thiocyanate as a protein denaturant¹⁸ and polyadenylated RNA was isolated by chromatography on poly(U)-Sephrose¹⁹. RNA samples were denatured with glyoxal, electrophoresed on a 1% agarose gel and transferred to nitrocellulose filters²⁰. The filters were hybridized with nick-translated probes¹⁶ for 40 h in 50% formamide, 5× SSC, 2× Denhardt's, 20 mM sodium phosphate, 0.1% SDS and 100 $\mu\text{g ml}^{-1}$ denatured DNA. Filters were then washed in 2× SSC, 0.1% SDS at 23 °C followed by 0.1× SSC, 0.1% SDS at 55 °C for 1 h. **a**, Purified DNA fragments from the *npt-II* (*Bam*HI/*Hind*III) and *hgh* (*Bam*HI/*Sma*I) genes labelled with approximately equal specific activities (10^8 c.p.m. μg^{-1}) were hybridized to a filter containing 0.5–2 μg poly(A)⁺ RNA from calli transformed with: lane 1, control pMON vector; lane 2, –46 35S promoter-*hgh*; lane 3, –105 35S promoter-*hgh*; lane 4, –168 35S promoter-*hgh*; lane 5, –343 35S promoter-*hgh*. **b**, Purified *npt-II* and *hgh* fragments were labelled so that the specific activity of the *npt-II* fragment was approximately 10-fold lower than that of the *hgh* fragment. Hybridization was to 2 μg poly(A)⁺ RNA from calli transformed with: lane 1, –46 35S promoter-*hgh*; lane 2, –105 35S promoter-*hgh*. **c**, The GH-Neo24 plasmid described in Fig. 2 legend was used as the probe. Each RNA preparation was made from a pool of several plants derived from different transformation events, but carrying the same 35S promoter deletion fragment. Gel samples contain ~2 μg poly(A)⁺ RNA from: lanes 1–4, RNA from different parts of plants transformed with –168 35S promoter-*hgh*; lane 1, roots; lane 2, petals; lane 3, stems; lane 4, leaves; leaf RNA from plants transformed with: lane 5, –343 35S promoter-*hgh*; lane 6, –168 35S promoter-*hgh*; lane 7, –105 35S promoter-*hgh*; lane 8, –46 35S promoter-*hgh*.

Calli obtained by wounding were selected for kanamycin resistance (50 $\mu\text{g ml}^{-1}$) and hormone independence. Plants were regenerated from co-culture with selection for kanamycin resistance, making use of the short transfer property of the split-end vector system⁸ which eliminates the T-DNA tumour genes.

Southern blots of DNA extracted from transformed calli (Fig. 2) showed the expected 1.58-kilobase (kb) *Eco*RI fragment containing the NOS promoter-*npt-II* gene and a 1.7–2.0-kb *Eco*RI fragment containing the 35S promoter-*hgh* gene, the size depending on the length of the specific promoter deletion fragment involved, indicating that no rearrangements had occurred in these two genes. Comparison with reconstructions of gene copy numbers in lanes 5–7 indicate that the copy number of these two genes varies between different transformed calli, but that there is an equal number of the two genes in each callus,

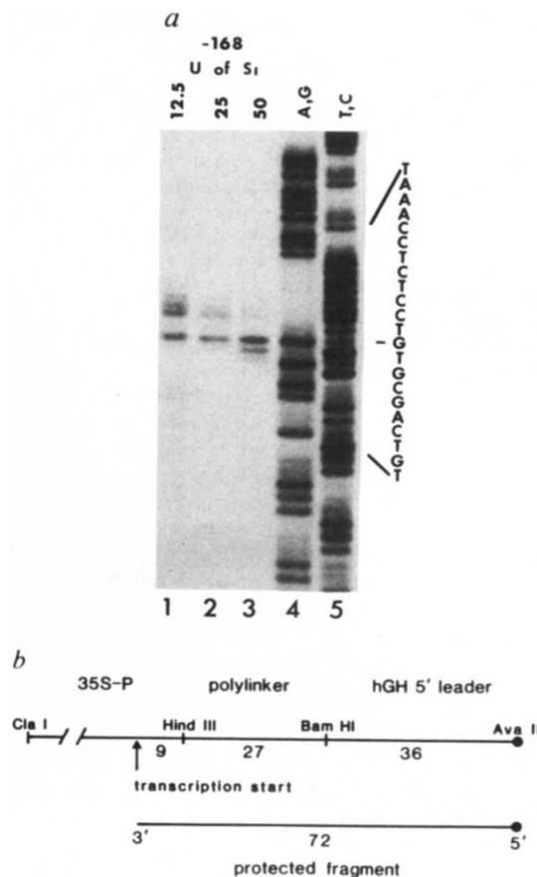


Fig. 4 **a**, *S*₁ nuclease mapping²¹ of the 5' end of the *hgh* transcript to the CaMV sequence. Total RNA (10 μg) from callus carrying the –168 35S promoter-*hgh* gene construction was hybridized to the probe diagrammed in **b** in 80% formamide at 37 °C. Samples were digested in 100 μl with the following amounts of *S*₁ nuclease: lane 1, 12.5 U; lane 2, 25 U; lane 3, 50 U. The same probe was sequenced using the Maxam and Gilbert technique²²: lane 4, A+G reaction; lane 5, T+C reaction. All samples were electrophoresed on an 8% polyacrylamide/7 M urea gel. The DNA sequence¹³ surrounding the *hgh* RNA 5' end is shown to the right. **b**, Diagram of *S*₁ probe and region protected by *hgh* RNA. A *Cla*I/*Ava*II DNA fragment extending from the 5' border of the 35S promoter fragment and into the 5' leader region of the *hgh* transcript was 5' end-labelled and strand separated on a polyacrylamide gel. Between the probe's labelled *Ava*II end and the 5' end of the *hgh* RNA are 72 base pairs of *S*₁-protected sequence.

supporting the assumption that the two genes are co-transferred into the tobacco genome. Thus, the NOS promoter-*npt-II* gene provides a control for variables inherent in the transformation system including copy number and integration site.

Northern blots of poly(A)⁺ RNA from transformed calli carrying each of the 35S promoter deletions showed the expected *npt-II* transcript and also a 2.3-kb transcript that contains *hgh* sequences (Fig. 3). The 35S promoter is responsible for transcription of the *hgh* RNA, as *S*₁ protection experiments indicate that the 5' end of this RNA maps to the normal 35S transcription start site (Fig. 4). All the deleted promoters initiate transcription at the same site (data not shown). To compare the transcriptional activities of the different deletions, the ratio of *hgh* to *npt-II* transcripts was determined for each RNA sample. By quantitating silver grains eluted from bands on X-ray films⁹, the *hgh/npt-II* ratios were: –46, 0.16/1; –105, 0.9/1; –168, 2.6/1; –343, 3.1/1. The *hgh/npt-II* ratio is the same for different RNA preparations from calli transformed with the same deletion fragment (data not shown). The –46 promoter fragment has an approximately 20-fold lower transcription level than the –343 fragment, whereas the –105 fragment has a 3-fold decrease and the –168 fragment has no significant decrease in activity. We also found

that the -343 fragment and a -941 fragment have no significant difference in promoter activity (data not shown).

Regenerated transformed tobacco plants contain one to two copies per genome of the NOS promoter-*npt-II* and 35S promoter-*hgh* genes, determined by Southern blots (data not shown). This low copy number in plants, compared with the high copy numbers in calli (Fig. 2), could be explained by the transformation procedure (co-cultivation versus wounding) or by the type of T-DNA transfer event involved (short versus long). Northern blots show that leaves of plants transformed with each of the four 35S promoter deletions contain the same 2.3-kb *hgh* RNA found in transformed calli (Fig. 3c). By S₁ analysis the 5' end of this leaf *hgh* RNA was found to be identical to that of the callus *hgh* RNA (data not shown). The effects of the deletions on promoter activity in transformed plant leaves (Fig. 3c) closely resemble results described for the transformed calli. The 35S promoter was also active in roots, petals and stems of transformed plants (Fig. 3c), with deletions having no specific effects on tissue expression (data not shown). The ratio of *hgh/npt-II* transcripts is constant in the different tissues. Both transcripts appear reduced in the root RNA preparation, but this could be due to varying amounts of ribosomal RNA contamination in the polyadenylated RNA preparations.

Here, we have shown that although the normal host range of CaMV is limited to members of the Cruciferae, the 35S promoter is active when isolated as a fragment from the viral genome and integrated into the tobacco genome. Thus, accurate transcription from the 35S promoter does not depend on any *trans*-acting viral gene products. The ability to regenerate tobacco plants from transformed protoplasts has allowed us to demonstrate that the 35S promoter is expressed in leaves, stems, roots and petals.

Promoter deletion analysis in transformed calli and plants showed that a -46 fragment, which does contain a TATA-box sequence (see Fig. 1), produces a low level of correctly initiated transcripts. The region between -46 and -105 which greatly increases the level of transcription contains a CAAT-box sequence, an inverted repeat region and a sequence resembling the consensus core for enhancers in animal systems (GTGG^{AAA}_{TTT}G) (ref. 10; see Fig. 1). We are investigating whether one or more of these features plays a substantial role in increasing the level of 35S promoter activity or could act to increase transcription from a foreign promoter.

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Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells

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Epstein-Barr virus (EBV) infects human B lymphocytes, transforming the infected cells into dividing blasts that can proliferate indefinitely (see ref. 1 for a review). The viral genome of 172 kilobase pairs (kbp) is a plasmid in most transformed cells²⁻⁴. We have identified a region of EBV DNA, termed *oriP* (nucleotides 7,333-9,109 of strain B95-8), which acts in *cis* to permit linked DNAs to replicate as plasmids in cells containing EBV DNA⁵. We have postulated the existence of a *trans*-acting gene allowing *oriP* function. Here we report that this gene lies in a 2.6-kbp region of the viral genome (nucleotides 107, 567-110, 176) which encodes the EBNA-1 antigen⁶⁻⁸. We show that circular DNAs containing *oriP*, the EBNA-1 gene and a selectable marker replicate autonomously in cells derived from at least four developmental lineages and from at least three species. We also find that the one-third of the EBNA-1 gene repetitive in sequence is not essential for the *trans*-acting function that EBNA-1 gives *oriP*.

To map the gene encoding the proposed *trans*-acting function, overlapping segments of the EBV genome were first introduced individually into the human thymidine kinase (TK)-negative cell line 143, using a set of recombinant plasmids selected using the antibiotic G418 (ref. 5). These G418-resistant cell lines were transfected subsequently with the hypoxanthine-aminopterin thymidine (HAT)-selectable plasmid pΔTK or its derivative, pTKBamC, which contains *oriP*. Only those G418-resistant cells that carried EBV DNA mapping from *Bam*HI-Z to *Sal*I-F (Fig. 1a, b) supported pTKBamC as a plasmid. Three of four 143 clones carrying pBamZRSalF (termed 143/BamZRSalF) could be transfected stably 5-30-fold more efficiently with the *oriP*-containing plasmid, pTKBamC, than with its parent, pΔTK (Table 1, experiment 1). Analysis of DNA from the HAT-resistant clones showed that the 143/BamZRSalF cell lines efficiently transfected by pTKBamC contained it as a plasmid at two to four copies per cell (Table 1). 143 clones carrying all other regions of the EBV genome showed no increase in transfection frequency-dependent on *oriP* and did not contain pTKBamC as a plasmid. These results imply that the EBV DNA spanning the *Bam*HI-Z/*Sal*I-F fragment encodes the proposed *trans*-acting product which allows maintenance of *oriP*-bearing plasmids.

The only viral product encoded in this region of the genome and known to be expressed in EBV-transformed cells is the nuclear antigen EBNA-1. It is encoded in the *Bam*HI-K fragment, which lies within the *Sal*I-F fragment of EBV DNA⁶⁻⁸. A 2.9-kbp *Bam*HI/*Hind*III subfragment of *Bam*HI-K encodes most, if not all, of the EBNA-1 polypeptide^{9,10} (see maps of Fig. 1). This 2.9-kbp subfragment was cloned into a G418-selectable plasmid containing the transcriptional enhancer of simian virus 40 (SV40), and the resulting plasmid, pSVoB-H2.9, was introduced into 143 cells. Two of five such cell lines, 143/SVoB-H2.9 clones 1 and 4, expressed levels of EBNA-1 that could be detected by anti-complement immunofluorescence (our unpublished observations). When we transfected pTKBamC into these two 143-derived clones, we efficiently selected HAT-resistant colonies in which one to three copies of the *oriP*-bearing plasmid were maintained per cell (Table 1). Thus, the *trans*-acting product required for *oriP* function maps in the 2.9-kbp region encoding EBNA-1. The SV40 enhancer was required for efficient expression of the *trans*-acting function from the integrated 2.9-kbp fragment (data not shown). This finding is consistent with the observation that the mRNA for this region is a 3.7-kb transcript beginning upstream of *Bam*HI-K^{11,12}.

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Table 1 Ability of 143 cells carrying integrated fragments of EBV DNA to maintain plasmids containing *oriP*

Cell line	No. of colonies after transfection with:		Plasmid molecules per cell:	
	p $\Delta\pi$ TK	ptKBamC	p $\Delta\pi$ TK	ptKBamC
Expt 1				
143/BamZRSaIF				
Clone 1	48	800	0	2
Clone 2	92	480	0	4
Clone 3	24	36	0	0
Clone 4	18	540	ND	ND
All others	18-190*	8-80*	ND	0
Expt 2				
143/BamZRSaIF				
Clone 2	3	410	ND	ND
143/SVoB-H2.9				
Clone 1	2	450	0	1.5
Clone 4	3	650	0	3
	pHyg	pHEBo2	pHyg	pHEBo2
Expt 3				
143/SVoB-H2.9				
Clone 4	3	6,000†	ND	3
143 control	5	15	ND	ND

Clonal, G418-resistant derivatives of 143 cells carrying integrated copies of the indicated plasmid were constructed as previously described⁵. Cell lines are named for the plasmid carried; for example, 143/BamZRSaIF carries pBamZRSaIF (ref. 5). pBamZRSaIF contains the EBV DNA shown in Fig. 1a, 1b. 'All others' refers to different 143 clones carrying each of nine plasmids that span the remainder of the EBV genome⁵. Four clonal lines carrying each integrated plasmid were tested. pSVoB-H2.9 was constructed by inserting the 2.9-kbp *Bam*HI/*Hind*III fragment of *Bam*HI K (Fig. 1) between the *Bam*HI site and the *Hind*III site of pKan2 (ref. 5) and by inserting the origin-containing *Hpa*II/*Hind*III fragment of SV40 between the *Cla*I site and the *Hind*III site of the resulting plasmid. Using the method of Graham and Van der Eb²⁰, $\sim 1 \times 10^6$ cells from each line were transfected with 2.5 μ g of plasmid DNA and selected after 24 h in medium containing HAT or 150 μ g ml⁻¹ hygromycin B (Calbiochem). The HAT-selectable vector p $\Delta\pi$ TK carries the *tk* gene of herpes simplex type 1 (see the construction of pKan2 in ref. 5). pTKBamC is p $\Delta\pi$ TK carrying the *oriP*-containing *Bam*HI-C fragment of EBV. pHEBo2 confers resistance to the drug hygromycin B¹³ and carries *oriP* (ref. 14 and Fig. 1). pHyg is identical to pHEBo2 except that it lacks the EBV sequences. Selected colonies were amplified and analysed for the presence of plasmid molecules as shown in Fig. 2. The limit of detection was 0.2 copies per cell. In addition, pTKBamC was recovered from DNA of transfected 143/BamZRSaIF clones 1 and 2 by transformation of *E. coli* and shown not to be rearranged, as described previously⁵. For 'all others', a single HAT-resistant clone from one line carrying each region of EBV DNA was analysed. Using a probe specific for the *oriP* region, each of these was shown to carry integrated copies of pTKBamC. ND, not determined.

* Although the transfection efficiency of these lines varied by more than 10-fold, the ratio of the number of colonies obtained with pTKBamC to the number obtained with p $\Delta\pi$ TK was between 0.3 and 0.8 for every cell line.

† The fraction of 143/SVoB-H2.9 clone 4 cells that can be stably transfected with pHEBo2 is 0.5-1%, close to the 1-2% that can be transfected with SV40 DNA to express T antigen transiently.

We constructed plasmids carrying *oriP*, the *EBNA-1* gene and a selectable marker and found that they replicated autonomously in a variety of cultured cells. Because many cell lines are resistant to high concentrations of G418, we first made the *oriP*-bearing vector pHEBo2, which confers resistance to the drug hygromycin B^{13,14} (Fig. 1). pHEBo2 was >100-fold more efficient in conferring drug resistance to 143 cells expressing EBNA-1 than to the same number of control cells (Table 1, experiment 3). A similar effect of the *EBNA-1* gene was observed when it was inserted in pHEBo2. Five different derivatives of pHEBo2 carrying the *EBNA-1* gene yielded hygromycin B-resistant colonies 10-100 times more efficiently than did pHEBo2 when transfected into HeLa cells; all five were maintained as plasmids in the resistant cells (Fig. 1b).

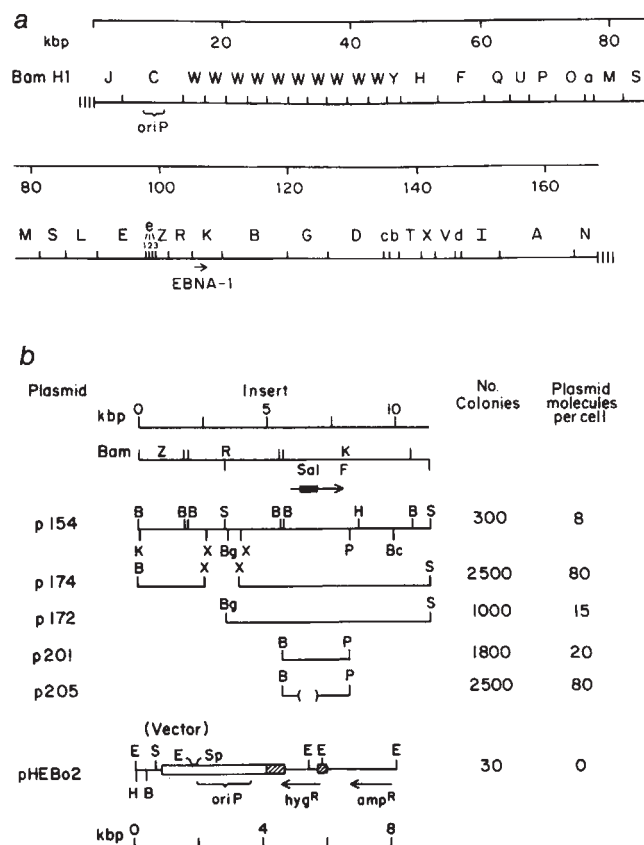


Fig. 1 a, Map of linear, Epstein-Barr viral DNA indicating all but the smallest *Bam*HI restriction endonuclease fragments¹⁰ and the positions of *oriP* and the *EBNA-1* gene. The vertical lines represent the terminal repetitions found in the viron DNA. b, Structure and function of plasmids carrying *oriP* and the *EBNA-1* gene. The vector pHEBo2 is shown at the bottom, linearized at the *Eco*RI site derived from pBR322. Its construction is described elsewhere¹⁴. The bacterial gene for hygromycin B resistance¹³ is expressed using the promoter and polyadenylation signals of the herpes simplex *tk* gene (hatched boxes). *OriP* is present on an *Sst*I/*Sst*II fragment of EBV DNA⁵ (open box). The relevant EBV *Bam*HI and *Sal*I fragments are shown at the top. The arrow represents the open reading frame BKRF1¹⁰ (*EBNA-1*), with the thickened part representing the triplet repeats. In the middle of the figure, plasmids are represented by the DNA segment inserted into pHEBo2. Inserts of the first three plasmids are between the *Bam*HI and *Sal*I sites of pHEBo2. For p201 and p205, the DNA was inserted by blunt-edged ligation between *Hind*III and *Sph*I sites. The orientations of all inserts relative to the vector are as shown. p174 was derived from p154 by deletion of the DNA between the *Xho*I sites shown. The insert of p205 carries a 700 ± 20-bp deletion at the site of the triplet repeats. The triplet repeat array is 717 bp long in the B95-8 strain¹⁰ from which the *EBNA-1* gene was cloned. The deletion was obtained by subcloning from the Ch4A clone EB 62-71 in which this region spontaneously deletes¹⁵. We have not determined the sequence at the boundaries of the deletion, but note that deletion of the triplet repeats by homologous recombination would preserve the reading frame. A similarly obtained deletion was reported to be in frame⁹. All restriction enzyme cleavage sites relevant to this report are indicated for the insert of p154. B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pvu*II; S, *Sal*I; Sp, *Sph*I; X, *Xho*I. **Methods.** Plasmids were tested by transfecting 1×10^6 HeLa cells as described in Table 1 legend and selecting for resistance to hygromycin B at 150 μ g ml⁻¹. Resistant colonies were counted after 11-21 days of selection, trypsinized and carried or expanded for 18-23 population doublings. The amount of free plasmid present in the cell populations was determined as shown in Fig. 2a, except that DNAs were usually not digested with endonucleases. The average of determinations from at least two independent transfections is shown.

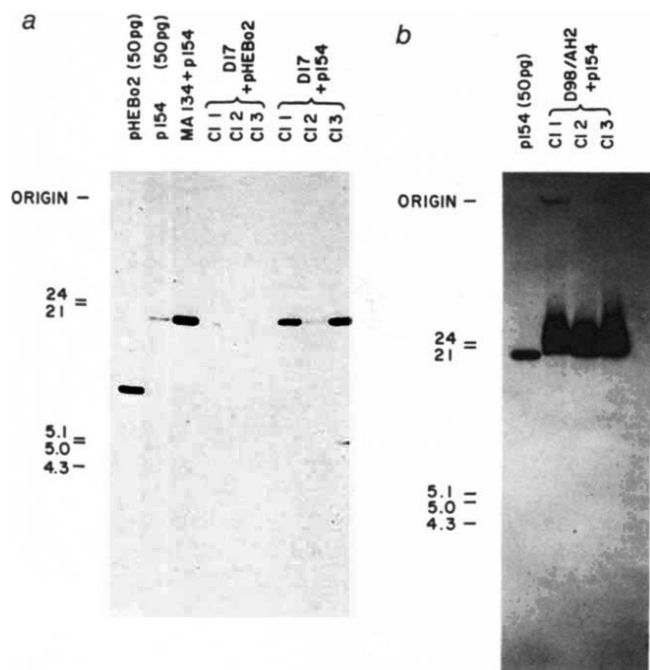


Fig. 2 Analysis of EBV-derived plasmids in transfected cells. DNAs were separated by electrophoresis in 0.5% agarose and detected by the method of Southern²⁷ using the plasmid pHyg as probe, labelled isotopically to a specific activity of $2-4 \times 10^8$ c.p.m. μg^{-1} . **a**, DNA was isolated from supernatants of Hirt extracts²⁸ of 2×10^6 hygromycin B-resistant MA134 cells (a pool of 34 clones) or D17 cells (individual clones) transfected with the indicated plasmids. DNAs were linearized by cleavage with the following enzymes: MA134 or D17 plus p154 with *Bcl*I, D17 plus pHEBo2 with *Hind*III, pHEBo2 standard with *Hind*III, and p154 standard with *Kpn*I. Cleavage of p154 DNA isolated from the MA134 and D17 cells by *Bcl*I shows that the plasmid had lost its methylation, and hence, had replicated. **b**, Total DNA from 1×10^6 cells of three D98/AH2 clones carrying p154 was analysed after digestion with *Bgl*II. A long exposure of the autoradiogram (96 h) is shown to demonstrate the absence of any additional bands which could result were vector sequences integrated. This experiment does not eliminate the possibility that these lines could carry integrated fragments of p154 which lack homology to pHyg. The positions and sizes in kbp of DNA size markers, λ DNA cut with *Eco*RI and *Hind*III, are indicated.

Table 2 Ability of EBV-derived replicons to function in cells of various origins

Cell line	Plasmid	Frequency of stable transfection	Plasmid molecules per cell
Human			
D98/AH2	p154	1×10^{-4}	7,12,14
143	p172	2×10^{-4}	1,10
143	pHEBo2	5×10^{-5}	0
293	p154	1×10^{-4}	35
293	pHEBo2	2×10^{-5}	0
Wilson	p174	3×10^{-6}	10, 16
Wilson	pHEBo2	$< 2 \times 10^{-7}$	—
K562	p172	2×10^{-5}	9,27,90
Monkey			
MA134	p154	3×10^{-5}	5
MA134	pHEBo2	$< 5 \times 10^{-7}$	—
Dog			
D17	p154	6×10^{-4}	1.5, 5, 5
D17	pHEBo2	2×10^{-4}	0
Rodent			
BALB/c3T3	p174	1×10^{-4}	0*
BALB/c3T3	pHEBo2	1×10^{-4}	0
HTC-A	p172	9×10^{-4}	0
V79	p174	9×10^{-4}	0, 0, 0*

Plasmid DNAs (Fig. 1b) were introduced by the calcium phosphate method for all cell lines except K562 and Wilson, for which fusion with *E. coli* spheroplasts²¹ and electroporation²², respectively, were used as described elsewhere¹⁴. Transfected cells were selected with the following concentrations of hygromycin B ($\mu\text{g ml}^{-1}$): D98/AH2 (ref. 23), 143 (ref. 24), 293 (ref. 25), BALB/c3T3 and MA134, 150; D17 (obtained from American Type Culture Collection), 200; Wilson (obtained from Dr Ian McGrath), K562 (ref. 26) and V79, 300; HTC-A (obtained from Dr H. Pitot), 500. < indicates that no resistant clones appeared within the number of cells tested. No resistant colonies arose from any cell line without transfection. To determine the number of plasmid molecules per cell, individual colonies or pools of colonies were amplified and their DNAs analysed as in Fig. 2, using pHEBo2 DNA as a probe. Limits of detection were ≤ 0.2 copies per cell. Where pools of colonies were analysed, a single value is given. Where individual colonies were analysed, the value obtained for each clone is given.

* These resistant colonies, obtained from the rodent cell lines, were shown to carry integrated vector sequences. In addition, BALB/c3T3 cells carrying integrated pSVoB-H2.9 and expressing levels of EBNA-1 detectable by anti-complement immunofluorescence could not maintain the *oriP*-bearing plasmid pHEBo2 (limit of detection 0.06 copies per cell).

That the smallest of these plasmids, p201 and p205, work efficiently implies that the *trans*-acting function required by *oriP* maps entirely in a 2.6-kbp *Bam*HI/*Pvu*II fragment and does not include all of the *EBNA-1* coding sequences (Fig. 1b). Between the *Bam*HI site and the *Pvu*II site lies an open reading frame of the correct size to encode the EBNA-1 polypeptide¹⁰. About one-third of this open reading frame is a repetitive sequence of glycine and alanine codons which encodes part of EBNA-1^{7,8} (arrow in Fig. 1b). A spontaneous deletion of the repetitive region of the *EBNA-1* gene was obtained from a recombinant bacteriophage clone grown in *recA*⁺ *Escherichia coli*¹⁵. If deletion of the triplet repeats occurred by homologous recombination, the reading frame would be preserved. A similar, spontaneous deletion was observed by Fischer *et al.* to be in-frame⁹. We found that p205, which carries this deletion, replicates more efficiently than p201 (Fig. 1b), indicating that most or all of the triplet repeats are dispensable for the *trans*-acting function that the *EBNA-1* gene product gives *oriP*.

These experiments do not imply that transcription of the *EBNA-1* gene on any of the described plasmids occurs as it does from the viral genome. If the *EBNA-1* gene is placed on pHEBo2 in the opposite orientation to that shown in Fig. 1b, it fails to function unless a transcriptional enhancer is provided

(data not shown). The expression of the *trans*-acting function obtained with plasmids such as p201 may depend on a promoter(s) present on pHEBo2, perhaps one of those found near the bacterial ampicillin resistance gene^{16,17}.

EBV-derived replicons, plasmids carrying both *oriP* and the *EBNA-1* gene, have been introduced into a variety of cell lines (Table 2). Figure 2 shows the ability of these plasmids to replicate in human D98/AH2 cells (of epithelial origin), MA134 African green monkey kidney cells and D17 dog fibrosarcoma cells. pHEBo2, which lacks the *EBNA-1* gene, was not found as a plasmid in the D17 cells (Fig. 2a). Plasmid p154, which is pHEBo2 plus the *EBNA-1* gene on an 11-kbp insert, was present as a plasmid at about five copies per cell in a pool of hygromycin B-resistant clones of MA134, at 1.5–5 copies per cell in three clones of D17, and at 7–14 copies per cell in three clones of D98/AH2. Cell clones carrying p154 as a plasmid generally do not have vector sequences integrated into the host chromosomes (Fig. 2b). In addition to functioning in human cells of epithelial and fibroblast origin, EBV-derived plasmids were found to be maintained in Wilson cells, an EBV-negative, human B-lymphoma cell line, and in K562 cells, a human erythroleukaemia cell line, but not in three cell lines from rodents (Table 2).

The rate of loss of the EBV-derived replicon, p154, in populations of D98/AH2 grown in the absence of selection was determined using a clonal assay described previously⁵. In the three clones carrying p154 analysed in Fig. 2b, the plasmid was lost at rates of 2, 3 and 5% per generation (measured at 18 and 27 generations after removal of the drug). The loss of hygromycin resistance in dividing cells in these three populations is consistent with the absence of integrated copies of the plasmid (Fig. 2b). This rate of loss is the same as that for *oriP*-bearing plasmids in cells containing EBV DNA⁵. Thus, the *EBNA-1* gene permits the maintenance of *oriP*-bearing plasmids as efficiently as does all of EBV DNA.

The wide host range and maintenance of the EBV-derived plasmids is in marked contrast to the replication of plasmids derived from BPV and SV40; BPV-derived plasmids have not been reported to replicate in cell lines derived from species other than mouse or rat, and those derived from SV40 are not usually maintained as plasmids¹⁸. For the human, monkey and dog cell lines mentioned here, we have not observed integration of plasmids carrying *oriP* and a functional *EBNA-1* gene. There is no reason to believe that such plasmids cannot be integrated into host chromosomes, however, as the entire EBV genome has been found integrated in more than one instance¹⁹. Our results indicate that EBV-derived replicons are more often maintained as plasmids than as integrated molecules in the non-rodent cells we have studied. That EBV-derived plasmids can be maintained in a variety of mammalian cells makes them useful for introducing genes into these cells and for isolating molecular clones of genes from these cells. In particular, plasmids containing both *oriP* and the *EBNA-1* gene should be essential vehicles for identifying and analysing those viral genes that, in addition to *oriP* and *EBNA-1*, are needed to transform human B lymphocytes.

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Gene deletion and restriction fragment length polymorphisms at the human ornithine transcarbamylase locus

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Deficiency of ornithine transcarbamylase (OTC; EC 2.1.3.3), a hepatic mitochondrial enzyme involved in the detoxification of ammonia^{1,2}, is a severe inborn error of metabolism. It is an X-linked disorder²⁻⁴ which results characteristically in ammonia intoxication, protein intolerance and mental retardation. Early death of affected hemizygous male infants is common, while clinical manifestations in heterozygous females are variable due to random X-chromosome inactivation²⁻⁵. Prenatal diagnosis by amniocentesis has not been feasible because OTC is not expressed in amniocytes and because no unusual metabolites can be detected in amniotic fluid. Fetal liver biopsy has been performed for some families at risk⁶, but the dangers inherent in this procedure severely limit its usefulness. In this report, we describe the use of a nearly full-length cloned human cDNA⁷ to begin to characterize normal and mutant human OTC genes. One of 15 affected males was found to have a partial deletion of the OTC gene. Two distinct restriction fragment length polymorphisms (RFLPs) were identified at the OTC locus using the restriction endonuclease *MspI*; 69% of women tested were heterozygous for one or both polymorphisms. Identification of these common polymorphisms makes it possible to offer prenatal diagnosis to a large fraction of obligate carriers and to provide information on carrier status to some females at risk.

In an initial experiment, high relative molecular mass (M_r) DNA from two control females was digested independently with restriction endonucleases *BglI*, *EcoRI*, *SstI*, and *BamHI* and analysed by DNA blot hybridization using the OTC cDNA as probe. No differences between the two controls were detected using these four enzymes. The total size of the gene, estimated by addition of the fragment lengths obtained with any one enzyme, is at least 25-30 kilobases (kb). In a further experiment, the hybridization intensities of all bands showed a clear X-chromosome dosage effect when DNAs from cell lines containing one, two, or four X chromosomes were analysed, indicating that all of the hybridizing fragments are localized to the X chromosome (not shown). The possibility that there are OTC pseudogenes on the X chromosome has not been eliminated.

Figure 1 depicts the hybridization patterns following *HindIII* digestion of DNA from seven OTC-deficient males. The pattern from the DNA of six of the patients is indistinguishable from that of controls, whereas the pattern from the DNA of one patient (lane 6) does not include the 3.1-kb band. Digestion of this patient's DNA with five other restriction endonucleases indicated that one hybridizing band was absent with each enzyme; no new bands were detected (not shown). This partial deletion has been localized to the 3' portion of the gene, based on hybridization experiments with 3'-fragments of the OTC cDNA (not shown). To date, we have examined DNA from 15 OTC-deficient males and have identified only this one with a gene deletion.

Because a substantial proportion (up to one-third) of cases of an X-linked lethal disorder may be due to new mutations⁸, we examined the DNA from the mother and maternal aunt of patient 6 to determine if they were carriers for this deletion. Figure 2a shows the middle portion of a blot of *EcoRI*-digested DNA from two control females (lanes 1, 2), the maternal aunt

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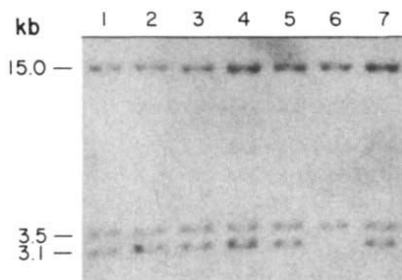


Fig. 1 Southern blot analysis of DNA from OTC-deficient males. High M_r DNA was prepared from peripheral blood lymphocytes or cultured skin fibroblasts by established procedures¹². Briefly, the lymphocytes were collected by centrifugation of whole blood, removal of plasma by aspiration and lysis of red blood cells with sterile water. The lymphocytes were pelleted and digested in 1% SDS-proteinase K (Boehringer-Mannheim, 100 $\mu\text{g ml}^{-1}$) at 50 °C for 3 h. Cultured cells were washed in phosphate-buffered saline before collecting by SDS-induced lysis and digestion under the same conditions. The solution was phenol extracted, and the nucleic acids were ethanol precipitated, dissolved and treated with RNAase A (Sigma, 100 $\mu\text{g ml}^{-1}$) at 37 °C for 3 h. The solution of high M_r DNA was then phenol extracted and dialysed extensively before use. Five μg DNA were digested with *Hind*III at 10 units per μg DNA; the digests electrophoresed in 0.8% agarose gels and UV-nicked, single-stranded DNA fragments were transferred to nitrocellulose by the method of Southern¹³. The filters were baked at 80 °C for 2 h in vacuo and incubated at 65 °C for 6–20 h in 0.75 M NaCl, 0.075 M sodium citrate, 5 $\times$ Denhardt's solution, 0.05 M sodium phosphate buffer, pH 6.5, 100 $\mu\text{g ml}^{-1}$ sheared, denatured salmon sperm DNA, 100 $\mu\text{g ml}^{-1}$ yeast RNA, and 0.001 M EDTA. Hybridization was performed using the same mixture, with the addition of 7% dextran sulphate and 4×10^7 c.p.m. of ^{32}P -labelled denatured probe, at 65 °C for 20 h. The probe, plasmid pHO731 containing the nearly full-length human cDNA for OTC⁷ (200 ng) was labelled by nick-translation¹⁴ using 50 μCi of [α - ^{32}P]dCTP (3,000 Ci mmol⁻¹, Amersham) and 5 units of *Escherichia coli* polymerase I (Kornberg polymerase, Boehringer-Mannheim) with no addition of DNase. The filters were then washed and exposed at -70 °C to XAR-5 film with intensifying screens.

(lane 3), the mother (lane 4) and the proband (lane 5). The band of interest (3.7 kb) is missing in the proband. In controls and in the maternal aunt, this band always has a stronger signal than that of the 3.4-kb band whereas in the mother's pattern, the signal from the 3.7-kb band is about equal in intensity to that of the 3.4-kb band. Therefore, the mother can be designated as a carrier for the deletion and the aunt, whose pattern is indistinguishable from that of controls, presumably is not a carrier. Using *Msp*I digests, a similar decrease in signal intensity is observed for a 17.5-kb band from the mother's DNA (Fig. 2b, lane 3) when compared with that from a control female (Fig. 2b, lane 1). This band is absent in the lane from her son's DNA (Fig. 2b, lane 2).

In this family (and any subsequent ones with major gene rearrangements), prenatal diagnosis will be feasible by qualitative analysis of the DNA of a male fetus, that is, by the presence or absence of specific band(s) in a Southern blot. Carrier detection will be possible in these families on a similar basis, as long as the rearrangement or deletion produces a new hybridizing band(s). In cases in which no new band is detected (see the family in Fig. 2) carrier detection will require a quantitative analysis of the intensity of the hybridization signal of the band(s) of interest. Clearly, caution must be exercised when performing such dosage blotting for diagnostic purposes.

The fact that only one OTC-deficient male with a gene deletion has been identified in this study suggests that most of the clinically significant mutations at this locus are minor deletions/insertions or single base substitutions, as reported for the β -thalassaemias⁹. In the majority of these cases, RFLPs, variations in DNA fragment lengths which result from DNA base

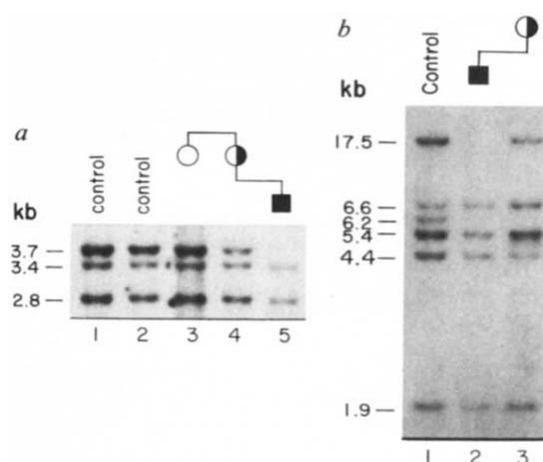


Fig. 2 Southern blot analysis of DNA from an OTC-deficient male with a gene deletion and from his maternal relatives. **a**, 3 μg DNA from two control females (lanes 1, 2), the maternal aunt (lane 3), the mother (lane 4), and the proband (lane 5) were digested with *Eco*RI and analysed by Southern blotting (see Fig. 1 legend). Only the middle portion of the blot is shown for comparison of hybridization intensities of the band of interest (3.7 kb) which is missing in the proband. **b**, 3 μg DNA from a control female (lane 1), the proband (lane 2), and his mother (lane 3) were digested with *Msp*I and analysed as described in Fig. 1 legend. Ethidium bromide staining of the gel (not shown) indicates that equal amounts of DNA are present in each lane. The DNA of the proband is missing a 17.5-kb band; all other differences in the restriction patterns between lanes are RFLPs (a band at 5.1 kb in lane 3 is reproduced poorly).

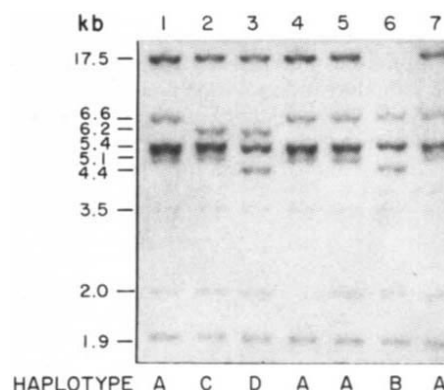
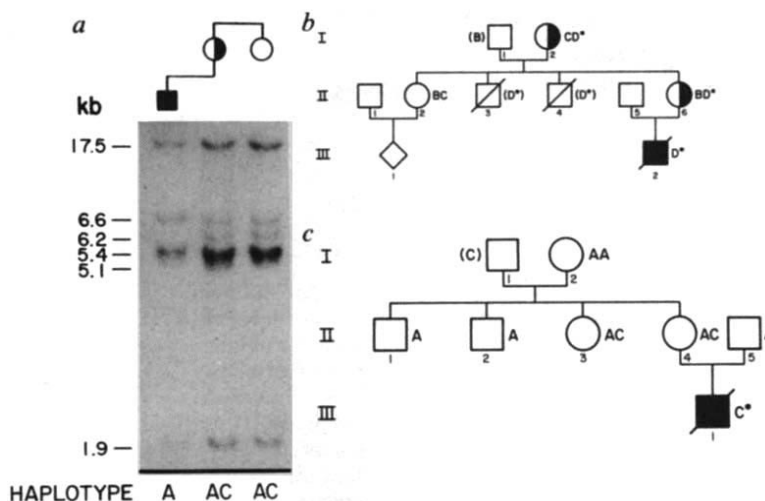


Fig. 3 Haplotype analysis of RFLPs revealed by *Msp*I digestion. 5 μg DNA from seven OTC-deficient males (same individuals as in Fig. 1) were digested with *Msp*I and analysed by Southern blotting as described for Fig. 1. The haplotype designations are: **A**, 6.6- and 5.1-kb bands; **B**, 6.6- and 4.4-kb bands; **C**, 6.2- and 5.1-kb bands; and **D**, 6.2- and 4.4-kb bands. The patient in lane 6 has a gene deletion (see Fig. 2 and text).

changes at restriction endonuclease recognition sites¹⁰, can be used diagnostically to detect the presence of the mutant gene.

We have found two different RFLPs at the OTC locus using the enzyme *Msp*I (Fig. 3). DNA from the seven affected males discussed in Fig. 1 yields five invariant bands, at 17.5, 5.4, 3.5, 2.0 and 1.9 kb (the bands at 3.5 and 2.0 kb are sometimes not observed under more stringent washing conditions). The variant bands in one polymorphic set are 6.6 and 6.2 kb in length; the bands in the second set are 5.1 and 4.4 kb. The hybridization pattern from the DNA of each male contains only one band from each polymorphic set. We have assigned haplotypes to the four possible combinations: an X chromosome with haplotype **A** shows the 6.6- and 5.1-kb bands; **B**, the 6.6- and 4.4-kb bands; **C**, the 6.2 and 5.1 kb bands; and **D**, the 6.2 and 4.4 kb bands. All four possible haplotypes (Fig. 3) are represented in the blot from the seven OTC-deficient males. In family studies (not

Fig. 4 Haplotype analysis in families affected with OTC deficiency. *a*, Haplotype analysis of women in an OTC family. Three μ g DNA from a male with OTC deficiency (lane 1), his mother (lane 2) and his maternal aunt (lane 3) were digested with *Msp*I and analysed as described in the Fig. 1 legend. *b*, Carrier evaluation by haplotype exclusion in an OTC family. Three μ g DNA from the affected male (individual III-2), his mother (individual II-6), his maternal aunt (individual II-2) and his grandmother (individual I-2) were digested with *Msp*I and analysed as described in Fig. 1. Haplotypes were assigned as indicated in the Fig. 3 legend. The haplotypes in parentheses were inferred from the haplotypes of other members of the family and from clinical information; (*) refers to the haplotype of the X chromosome which presumably carries the OTC mutation. *c*, Carrier evaluation by assignment of a spontaneous mutation. Three μ g DNA from an affected male (individual III-1), his mother (individual II-4), his father (individual II-5), his maternal uncles (individuals II-1, II-2) and his maternal aunt (individual II-3) were digested with *Msp*I and analysed as described in Fig. 1. Haplotypes were assigned as discussed in Fig. 3 legend. The parentheses indicate that the haplotype was inferred from the other haplotypes in the pedigree.



shown) they segregate in a mendelian fashion. The frequency of each haplotype has been evaluated by analysis of DNA from 35 unrelated control women. The frequency of the A haplotype is 0.45; B, 0.16; C, 0.28; and D, 0.11. Therefore, ~69% of women have different *Msp*I-detectable haplotypes on their two X chromosomes, a finding that potentially can provide prenatal diagnosis and carrier detection in families at risk.

All women who are (1) heterozygous for RFLP haplotypes and (2) obligate carriers for OTC deficiency are candidates for prenatal diagnosis. Figure 4a depicts the *Msp*I blot analysis of DNA from an OTC-deficient male (lane 1), his mother (lane 2) and his maternal aunt (lane 3). The mother, shown to be an obligate carrier for OTC deficiency by a protein tolerance test¹¹, is heterozygous for the A and C haplotypes. Because the proband's DNA shows the A haplotype, the OTC mutation must reside on the maternal X chromosome with the A haplotype. We predict that all future sons with the A haplotype will be affected and all sons with the C haplotype will be unaffected. Each daughter who inherits the X chromosome with the A haplotype from her mother will, similarly, be a carrier for OTC deficiency. The maternal aunt (lane 3) also has A and C haplotypes but her OTC carrier status is undefined because a spontaneous mutation might have occurred in her sister's chromosome marked by the A haplotype. Nonetheless, the haplotypic analysis is partially informative because, at the least, all the aunt's sons with the C haplotype will be unaffected.

Also, it may be possible to assess carrier status by haplotype exclusion, as shown in the pedigree in Fig. 4b. The maternal aunt of the proband, individual II-2, was referred to us for prenatal information, although her OTC carrier status was undefined. The grandmother, individual I-2, is presumably a carrier because two sons died in the neonatal period. Her X chromosome with the OTC mutation must have had the D haplotype, which was passed on to one of her daughters (individual II-6) and subsequently to the proband (individual III-2). Presumably the two maternal uncles also inherited the X chromosome with the D haplotype and, therefore, cannot be a carrier for OTC deficiency.

Carrier status can also be assessed by assigning a spontaneous mutation to a particular individual or generation, as indicated in the pedigree in Fig. 4c. The haplotype of the grandfather (individual I-1) was inferred based on the haplotypes of his

daughters. Because the proband (individual III-1) inherited the X chromosome with the C haplotype from his healthy grandfather via his mother, a spontaneous mutation must have occurred in either the mother's or proband's chromosome with that haplotype. Thus, the mother's OTC carrier status cannot be determined by this analysis. The maternal aunt (individual II-3) with haplotypes A and C is almost certainly not a carrier, however, because it is highly unlikely that another spontaneous mutation leading to OTC deficiency would have occurred in this pedigree.

Because the nature of the mutation in OTC deficiency is identifiable by DNA analysis in only a minority of patients, the identification of two common RFLPs at this locus is critical for providing prenatal information to obligate carriers for OTC deficiency and for assessing carrier status. The underlying assumption in each of these analyses is, of course, that the linkage of the mutation to a haplotype in a given family can be established unequivocally, usually by examining the DNA of an affected or an unaffected male family member.

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Domain of *E. coli* DNA polymerase I showing sequence homology to T7 DNA polymerase

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Escherichia coli contains three DNA polymerases that differ in their size, ability to interact with accessory proteins and biological function¹. Monomeric DNA polymerase I (Pol I) has a relative molecular mass (M_r) of 103,000 (103K) and is involved primarily in the repair of damaged DNA and the processing of Okazaki fragments; polymerase II is of M_r 120K, and polymerase III has a M_r of 140K, is responsible for the replication of the DNA chromosome and is just one of several proteins that are required for replication. DNA polymerases from bacteriophage as well as those of eukaryotic viral and cellular origin also differ with respect to their size and the number of associated proteins that are required for them to function in replication¹. However, the template-directed copying of DNA is identical in all cases. The crystal structure² of the large proteolytic fragment of Pol I shows that it consists of two domains, the larger of which contains a deep crevice whose dimensions are such that it can bind duplex DNA. The T7 polymerase consists of two subunits, the 80K gene 5 protein and the host-encoded 12K thioredoxin of *E. coli*^{3,4}. We show here that there is an amino acid sequence homology between at least eight polypeptide segments that form the large cleft in the Klenow fragment and polypeptides in T7 DNA polymerase gene 5 protein, suggesting that this domain evolved from a common precursor. The parts of the Pol I and T7 DNA polymerase molecules that bind the DNA substrate appear to share common structural features, and these features may be shared by all of these varied DNA polymerases.

The amino acid sequence of T7 DNA polymerase⁵ was compared with the sequence⁶ of the Klenow fragment using a program written by Peter Brick that followed the method described by McLachlan⁷. There were nine lengths of polypeptide in the Klenow fragment which showed significant amino acid sequence homology with segments of the T7 protein (Fig. 1). The sequences of the aligned segments are given in Fig. 2 and the lengths and number of identical residues in these regions are given in Table 1. The ends of the homologous stretches can be identified only approximately. The nine homologous segments vary in length from 9 residues to 45 residues and show 38–58% identical residues, with only a single amino acid deletion in two of the peptides. The lengths of the polypeptides that connect the aligned regions differ considerably in the two proteins and therefore cannot be easily compared.

Table 1 Comparison of Klenow fragment and T7 DNA polymerase

Region	Length	Identical	Fraction	Deletion
I	10	5	0.50	–
II	45	20	0.44	1
III	44	18	0.41	1
IV	12	7	0.58	–
V	14	6	0.43	–
VI	9	4	0.44	–
VII	12	4	0.33	–
VIII	21	8	0.38	–
IX	9	4	0.44	–
Total	176	76	0.43	2

Eight of the nine homologous segments are located in the C-terminal 400 residues of the Klenow fragment. The conserved regions comprise ~30% of the total Klenow sequence but make up 42% of the putative² DNA binding domain. The positions of the conserved segments in the C-terminal domain of the Klenow fragment are concentrated around the cleft that is proposed to bind DNA (Fig. 3). Six of the eight conserved regions border the DNA binding cleft and appear to cover most of its surface. Above the DNA binding cleft lies a 50-residue subdomain of the protein that is partially disordered in the electron density map. We have suggested² that this flexible subdomain may bind to the DNA, thereby closing off the cleft and allowing the protein to surround the bound DNA. The apparent flexibility of this subdomain may be necessary to allow the DNA duplex to have access to the cleft. The function of this domain may be to increase the processivity of the enzyme by slowing its rate of dissociation from DNA. Almost all of this subdomain (peptide segment II, Figs 2–4) can be aligned with a sequence in the T7 polymerase, with nearly 45% of the residues being identical. Thus, most of the Klenow structure that is thought² to be involved in binding the duplex DNA substrate appears to show a high degree of amino acid sequence conservation between these two proteins.

We find that the non-identical amino acids that lie within the conserved regions of polypeptide can be incorporated into the model of the Klenow fragment without severe steric or electrostatic consequences. Using the Evans and Sutherland picture system 300 (see ref. 8), we examined those amino acid positions within the conserved stretches at which there are non-identical residues in the two polymerases. Those residues that are charged and differ between the two enzymes all lie on the surface of the molecule. Those residues that lie in the interior of the Klenow fragment structure are either identical in the two proteins or conservative in the nature of their difference.

Between the eight conserved segments in the large domain lie nonconserved stretches of polypeptide, all in regions of less regular secondary structure and all containing a loop of polypep-

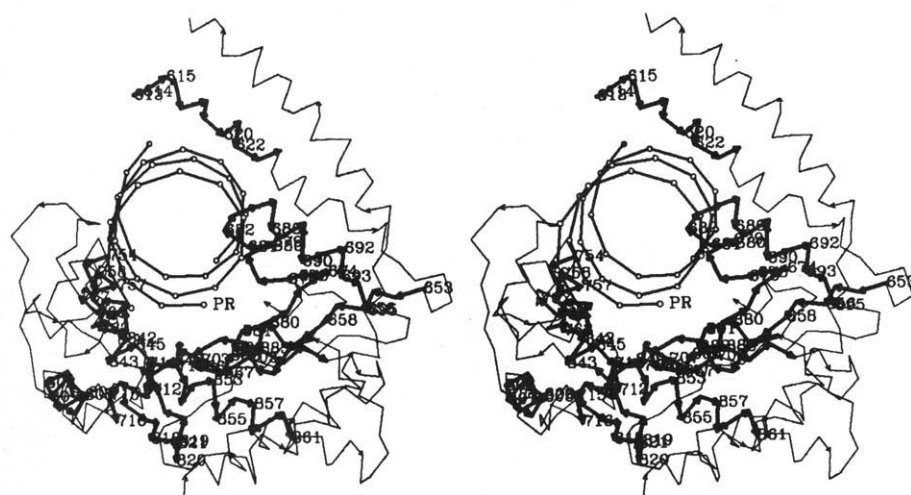


Fig. 1 Stereo drawing of the α -carbon backbone of the large domain of the Klenow fragment. Included is the DNA backbone of a duplex DNA substrate model built into the Klenow fragment. The eight stretches of polypeptide that appear to be homologous between the larger C-terminal domain of the Klenow fragment and T7 DNA polymerase are shown in heavier lines. These polypeptides form the interior surface of the cleft.

I	404 Leu-Leu-Lys-Pro-Leu-Leu-Glu-Asp-Glu-Lys Leu-Leu-Glu-Lys-Leu-Leu-Ser-Asp-Lys-His 181	413 190
II	578 Phe-Asp-Leu-Ser-Ser-Thr-Lys-Gln-Leu-Gln-Thr-Ile-Leu-Phe-Glu-Lys-Gln-Gly-Ile-Lys Phe-Asp-Pro-Ser-Ser-Arg-Asp-His-Ile-Gln-Lys-Lys-Leu-Gln-Glu-Ala-Gly-Trp-Val 334	588 344
	598 Pro-Leu-Lys-Lys-Thr-Pro-Gly-Gly-Ala-Pro-Ser-Thr-Ser-Glu-Glu-Val-Leu-Gln-Glu-Leu Pro-Thr-Lys-Thr-Thr-Asp-Lys-Gly-Ala-Pro-Val-Val-Asp-Asp-Glu-Val-Leu-Gln-Gly-Val 354	608 364
	618 Ala-Leu-Asp-Tyr-Pro Arg-Val-Asp-Asp-Pro 374	
III	653 Gly-Arg-Val-His-Thr-Ser-Tyr-His-Gln-Ala-Val-Thr-Ala-Thr-Gly-Arg-Leu-Ser-Ser-Thr Gly-Lys-Ile-His-Gly-Ser-Val-Asn-Pro-Asn-Gly-Ala-Val-Thr-Gly-Arg-Ala-Thr-His-Ala 414	663 424
	673 Asp-Pro-Asp-Leu-Gln-Asn-Ile-Pro-Val-Arg-Asn-Glu-Glu-Gly-Arg-Arg-Ile-Arg-Gln Phe-Pro-Asp-Leu-Ala-Gln-Ile-Pro-Gly-Val-Arg-Ser-Pro-Tyr-Gly-Glu-Gln-Cys-Arg-Ala 434	683 444
	693 Ala-Phe-Ile-Ala Ala-Phe-Gly-Ala 454	
IV	705 Asp-Tyr-Ser-Gln-Ile-Glu-Leu-Arg-Ile-Met-Ala-His Asp-Ala-Ser-Gly-Leu-Glu-Leu-Arg-Cys-Leu-Ala-His 475	715 485
V	754 Arg-Arg-Ser-Ala-Lys-Ala-Ile-Asn-Phe-Gly-Leu-Ile-Thr-Gly Arg-Asp-Asn-Ala-Lys-Thr-Phe-Ile-Tyr-Gly-Phe-Leu-Thr-Gly 518	764 529
VI	801 Tyr-Met-Glu-Arg-Thr-Arg-Ala-Gln-Ala Phe-Leu-Glu-Asn-Thr-Pro-Ala-Ile-Ala 555	
VII	814 Tyr-Val-Glu-Thr-Leu-Asp-Gly-Arg-Arg-Leu-Tyr-Leu Trp-Ile-Lys-Gly-Leu-Asp-Gly-Arg-Lys-Val-His-Val 592	824 602
VIII	841 Arg-Ala-Ala-Ile-Asn-Ala-Pro-Met-Gln-Gly-Thr-Ala-Ala-Asp-Ile-Ile-Lys-Arg-Ala-Met-Ile His-Ala-Ala-Leu-Asn-Thr-Leu-Leu-Gln-Ser-Ala-Gly-Ala-Leu-Ile-Cys-Lys-Leu-Trp-Ile-Ile 607	851 617
IX	878 Met-Gln-Val-His-Asp-Glu-Leu-Val-Phe Ala-Trp-Val-His-Asp-Glu-Ile-Gln-Val 650	

Fig. 2 Pol I and T7 polymerase sequence homologies.

tide that lies on the surface (Figs 1, 3). Each of the additions or deletions in the length of the polypeptide can be accommodated in the structure of the Klenow fragment. These loops could probably be modified without altering the conformation of the cleft. The amino-terminal half of the T7 DNA polymerase shows little homology to Pol I, with the exception of the 10-residue peptide I (Fig. 2). This domain and/or the non-identical peptides in the C-terminal domain could have different functions in T7 polymerase such as, for example, the interaction with other replication proteins.

The *polA6* mutation reduces the affinity of Pol I for DNA⁹. This mutation is a change in arginine 690 to histidine (C. Joyce, personal communication). The arginine lies on the second of a two-helix structure that protrudes into the cleft of the Klenow fragment and may be lying in the major groove of B-DNA. The amino acid sequence of this two-helix structure in the Klenow fragment is highly homologous with a sequence in T7 DNA polymerase (peptide VII, Fig. 2, Table 1) and includes an arginine in the position corresponding to Arg 690 in the Klenow fragment.

We have compared the amino acid sequences of DNA polymerase I and T7 DNA polymerase with the sequence of T7 RNA polymerase. We find that very short segments of polypeptide, corresponding to segments III, IV, V and VII, are weakly homologous between the DNA polymerases and a few short peptides that occur in T7 RNA polymerase. The regions of sequence that can be aligned are only 6-15 residues long and contain between three and seven identical residues. Thus, the possibility of an evolutionary relationship between the DNA and RNA polymerases cannot yet be evaluated.

We and others¹⁰ have compared the sequence of Pol I with various animal virus DNA polymerases and have found no significant sequence homology. Although this observation could result from eukaryotic polymerases having evolved indepen-

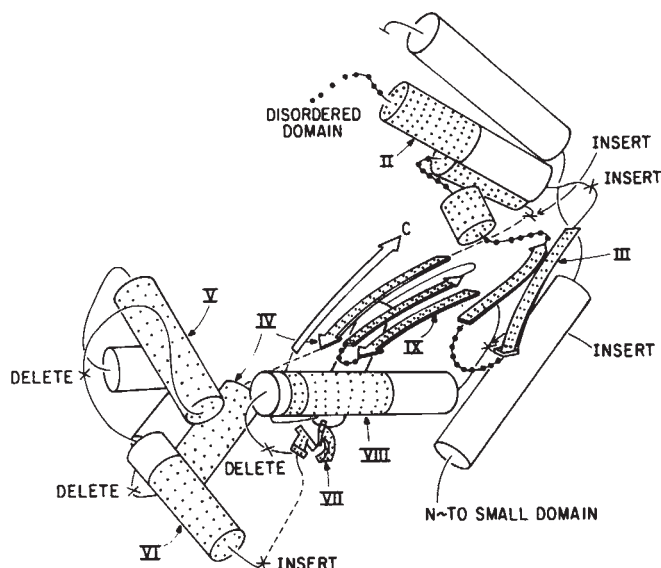


Fig. 3 Schematic drawing of the large domain of the Klenow fragment showing the regions that are homologous between the two polymerases as well as the locations of the necessary insertions and deletions. Homologous regions are dotted. Also indicated is the position of the 50-residue subdomain whose structure cannot yet be elucidated but whose sequence is highly homologous between the two polymerases.

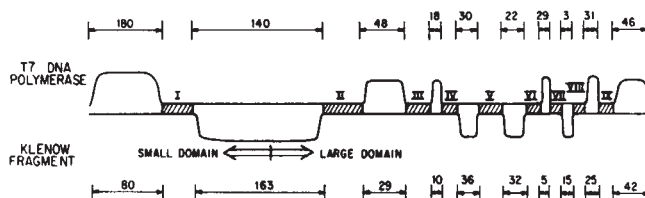


Fig. 4 Diagram showing the correspondence between the Pol I and T7 DNA polymerase sequences.

dently from a different precursor, it is also possible that the eukaryotic and prokaryotic polymerases have diverged so far from a common precursor that they have lost all trace of sequence homology. It is not uncommon to find proteins that contain two domains of nearly identical three-dimensional structure but which show no significant sequence homology, for example rhodanese¹¹. Also, comparison of myoglobin and haemoglobin sequences reveals examples in which fewer than 10% of the residues are identical even though the three-dimensional structures are closely similar¹². Thus, it may be necessary either to have sequences of polymerases from eukaryotic organisms that are more closely related to prokaryotic, or to obtain the structure of a eukaryotic polymerase to see whether or not all DNA polymerases have a domain that has essentially the same structure as the large domain of Klenow fragment involved in binding to DNA.

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Subduction regression of volcanism in New Zealand

IN discussing the origin of Cenozoic calc-alkaline volcanism in northern New Zealand, R. N. Brothers¹ uses a data base of 'time-lines' constructed from 93 selected K-Ar ages. These time-lines are said¹ to "represent the oldest dated rocks, and thus the initiation of volcanism, at eruptive centres throughout the northern North Island". We will show that this is untrue from which it follows that Brothers' time-lines are meaningless.

Brothers' oldest time-lines (in northern Northland) are 21 Myr. There is ample published evidence, however, of andesitic and basaltic tuffs and volcanoclastic sandstones in late Oligocene and earliest Miocene (~26–22 Myr) sedimentary sequences in northern New Zealand (Fig. 1)^{2–4}, which indicates considerably earlier initiation of volcanism within, or closely adjacent to, Northland, although the exact centres have not been determined.

Younger volcanic centres are identifiable; at least eight early Miocene centres are known in northern New Zealand and Brothers has based his time-lines on dated rocks from seven of them. The published stratigraphic data show clearly that eruptions began in the Middle–Upper Otaian Stage (early Miocene, ~23–21 Myr) in at least six centres spread along the length of Northland (North Cape, Whangarei Heads, Waipoua, North Coromandel, Waipoua, Waitakere, Auckland, Thames).

Hukaterere, Waitakere—Fig. 1). These dates are coincident with the oldest K-Ar dates available from a further two centres (Whangaroa¹, Tokatoka¹) which presumably also began erupting about the same time.

Brothers¹ agrees that eruption started at 21 Myr in identified centres in northern Northland, but bases his hypothesis of southward migration of volcanism on a 17-Myr time-line through Waitakere and north Coromandel. The 17-Myr date at Waitakere is taken from pillow lavas interbedded with fossil-dated (mid-Altonian) marine sediments that are high in the volcanic stratigraphic sequence^{5–7} (Fig. 1). The lowest volcanic breccias and lavas exposed at Waitakere are biostratigraphically dated as late Otaian (~21 Myr)⁵, but a number of submarine lahar deposits occurring within flysch around Auckland city are stratigraphically lower than exposed Waitakere rocks⁴. These lahars have features consistent with a western origin³ and indicate initiation of volcanism at Waitakere centre in the early half of the Otaian² (23 Myr). A similar submarine lahar deposit occurs within late Otaian flysch (~21 Myr), 60 km south of Auckland^{4,8}.

Brothers' 17-Myr time-line links Waitakere with dated plutons at north Coromandel⁹ which intrude the local volcanic sequence which in turn conformably overlies Otaian flysch¹⁰. Thus initiation of volcanism here is unlikely to have been later than 20 Myr. South of Brothers' 11-

Myr line (Thames), the base of the eastern volcanic pile in Coromandel Peninsula is not exposed^{11,12} and therefore none of the 'time-lines' between 11 Myr and 2 Myr can date the inception of volcanism in this eastern belt.

We have shown: (1) that the published stratigraphy, available to Brothers, indicates near-contemporaneous initiation of major volcanism in two NW–SE belts in northern North Island in the early Miocene (Otaian, ~23–21 Myr). This volcanism is calc-alkaline in the younger flows and intrusions¹³, and, although the geochemical character of the older volcanoclastics has not been determined, their petrography and geological setting are identical to the younger rocks. (2) The NE-trending 'time-lines', which form the data-base for Brothers' analysis of Cenozoic subduction and volcanism in northern New Zealand, do not record the earliest volcanism at most of the localities shown and are random lines connecting fortuitously-placed K-Ar dates. Therefore a tectonic model based on invalid 'time-lines' is itself invalid.

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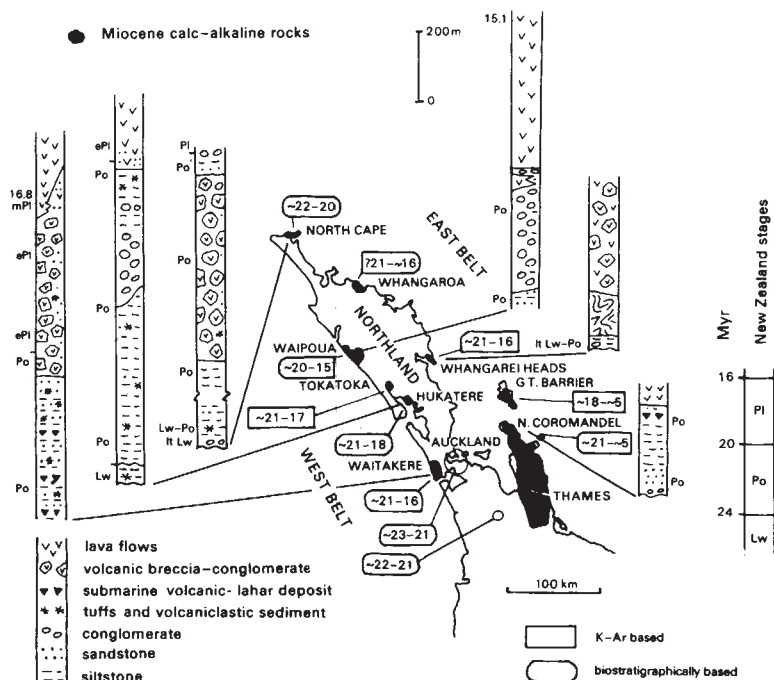


Fig. 1 The northern part of North Island, New Zealand, showing location and age of major centres of early Miocene calc-alkaline eruption. Absolute ages have been determined from a combination of K-Ar dates^{1,2} and converted biostratigraphic dates based on the International Time Scale model of Berggren¹⁴ (see right for conversion table from New Zealand stages¹⁵). Generalized columns summarize the stratigraphic evidence for the age of outbreak of volcanism in six areas, with position of biostratigraphic and K-Ar dates shown (North Cape^{16,17}, Whangarei Heads¹⁸, Waipoua^{2,19}, Hukaterere^{2,20}, Waitakere⁵, north Coromandel^{10,21}). New Zealand Stage symbols: Lw = Waitakian, Po = Otaian, Pl = Altonian.

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BROTHERS REPLIES—The comments by Ballance *et al.* reflect the problems of biostratigraphers trying to equate an imprecise time scale with the more exact ages provided by radiometric methods. The time-lines used in my study¹ were

based on K-Ar dates for dikes, flows and plutonic intrusions. It is these bodies, rather than volcanogenic sedimentary sequences, which define the locations of eruptive centres; in addition, they give more dependable ages for volcanism than data derived from biostratigraphy because volcanic detritus deposited by either air-fall or marine processes can travel considerable distances from its area of origin. For example, the age of the oldest sediments in the Waitakere centre is quoted by Ballance *et al.* from fossil evidence as late Otaian (~21 Myr) but these formations contain pebbles of hornblende diorite² and clasts of amphibole. There are no plutonic bodies in the Waitakere centre² where, moreover, the flows, sills and volcano-feeder dikes generally lack amphibole^{3,4}, so that this debris must have been introduced to the area. The regional geology^{5,6} clearly suggests a northwesterly source at the latitude of the Whangarei-Tokatoka time-line where amphibole-bearing volcanics and plutonics are present and where additional radiometric dates from the calc-alkaline suite now give ages as old as 27 Myr.

Biostratigraphical studies of the Waitakere² and other Northland centres^{7,8} and the observations made by Ballance *et al.* are inadequate because they ignore important petrological and volcanological aspects in sequences which are, essentially, volcanic accumulations. A number of deficiencies can be identified in their comments, including the following: (1) The geographically separate North Cape-Whangarei volcanic zone is unique within the North Island calc-alkaline suites and unusual even in the international geological record, in containing numerous andesites with garnets⁹ or high-pressure mineralogies¹⁰; the consanguineous nature and narrow regional extent of these rocks indicate an origin that is quite different from the common andesites of the North Island. (2) The Waipoua centre consists only of flood basalts¹¹ which should not be assigned to the same tectonic regime or comagmatic source as the andesite-diorite-dacite association to which the time-line concept and K₂O-SiO₂ indices have been applied¹. (3) A western source for the submarine lahars was suggested many years ago⁴, but this hypothesis needs to be proved from petrological affinities which have not yet been determined⁷ for these volcanic deposits. (4) In exposures in the Coromandel Peninsula, for volcanic edifices constructed in either an underwater or subaerial setting, rocks lying at high levels may be older in the sequence than units at lower sites near the apparent altitudinal base of the pile.

My summary¹ of calc-alkaline igneous activity in the North Island identified a mechanism for the Miocene-Holocene southeastward migration of volcanism, a pattern of eruption which has been described previously from K-Ar dates^{12,13}

and regional geology^{14,15} and is now supported by sea-floor spreading data¹⁶ and K₂O-SiO₂ indices¹. Ballance *et al.* do not provide a comparable body of evidence to justify their hypothetical concept of near-contemporaneous major volcanism in two NW-SE belts.

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GTP not cyclic GMP enhances secretion from permeabilized platelets

REPORTS by Knight and Scrutton¹ and from our laboratory² have shown that thrombin enhances the sensitivity to Ca²⁺ of the secretion of serotonin from permeabilized platelets. This effect was mimicked by adding a synthetic diacylglycerol, 1-oleoyl-2-acetyl-glycerol¹, and was closely associated with an enhanced formation of diacylglycerol in the permeabilized platelets². Diacylglycerol may

increase secretion of serotonin by activation of protein kinase C and by other mechanisms^{2,3}. Cyclic AMP blocked, and cyclic GMP enhanced, the stimulation of serotonin secretion by thrombin¹. This apparent example of reciprocal regulation by these cyclic nucleotides is in conflict with the results of experiments using intact platelets which indicated that both cyclic AMP and cyclic GMP have inhibitory effects⁴⁻⁶. Recent studies in our laboratory^{7,8} and the experiments cited below suggest that Knight and Scrutton¹ have misinterpreted their observations with cyclic GMP, which we believe are attributable to conversion of cyclic GMP to GTP.

Incubation of permeabilized platelets with ³H-labelled cyclic GMP under the same conditions as in our experiments on the secretion of platelet ¹⁴C-serotonin^{2,7}, led to the appearance of some GTP in the suspension medium and to almost complete conversion of the ³H-cyclic GMP in the platelet pellet to ³H-GDP and ³H-GTP (Table 1). It seems that cyclic GMP is metabolized almost as soon as it enters the platelets and that the GTP formed then diffuses out. Little ³H-GDP was found in the medium, suggesting that the ³H-GDP present in the platelets may be in a bound form. Addition of 50 μM 3-isobutyl-1-methylxanthine (IBMX) had relatively little effect on the metabolism of ³H-cyclic GMP by permeabilized platelets (Table 1), suggesting that the much lower concentrations used by Knight and Scrutton¹ to prevent breakdown of cyclic GMP were ineffective. IBMX (50 μM) did inhibit the secretion of ¹⁴C-serotonin from permeabilized platelets, but as this effect was observed in both the presence and absence of cyclic GMP, it cannot be attributed to inhibition of cyclic GMP breakdown. We have reported previously that low con-

Table 1 Metabolism of ³H-cyclic GMP by permeabilized platelets

Additions (other than ³ H-cyclic GMP and Ca ²⁺)	Fraction analysed	Distribution of ³ H recovered in guanine nucleotides (%)			
		Cyclic GMP	GMP	GDP	GTP
None	Supernatant	82	8	1	9
	Pellet	1	14	53	32
IBMX	Supernatant	88	5	0	7
	Pellet	4	14	50	32

Human platelets containing granule-bound ¹⁴C-serotonin were permeabilized and suspended in a Ca²⁺-free medium containing 5 mM ATP, as described in ref. 2. Samples (0.4 ml containing 0.33 × 10⁹ platelets) were equilibrated for 15 min at 0 °C with 0.1 ml of additions containing ³H-cyclic GMP (final concentration 10 μM), Ca²⁺ buffer giving a free Ca²⁺ concentration ([Ca²⁺]_{free}) of 1 μM and, where indicated, IBMX (final concentration 50 μM). These mixtures were incubated at 25 °C for 10 min, then platelets were isolated by centrifugation through silicone oil (density 1.023) at 12,000 g for 1 min and the supernatants and pellets were extracted with 10% (w/v) trichloroacetic acid (TCA). After removal of the TCA with ether and addition of carrier guanine nucleotides, these were separated by tlc in two dimensions on cellulose-coated plates (first solvent, *n*-butanol/acetone/acetic acid/14.8 M NH₃/water, 90:30:20:1:60 by vol.; second solvent, isobutyric acid/M NH₃/0.1 M EDTA, 125:75:2 by vol.). The guanine nucleotides were eluted with water and counted for ³H. In the starting material, 99% of ³H was contained in ³H-cyclic GMP and 1% in ³H-GMP. The secretion of platelet ¹⁴C-serotonin was measured in similar incubation mixtures with a final vol. of 0.1 ml (ref. 2); in the absence and presence of IBMX, serotonin secretion amounted to 9% and 6%, respectively, when cyclic GMP was omitted, and to 46% and 21%, respectively, when cyclic GMP was included.

Table 2 Effects of cyclic GMP and 8-bromocyclic GMP on the secretion of ^{14}C -serotonin from permeabilized platelets

Cyclic nucleotide added	Release of ^{14}C -serotonin (%)		
	With Ca^{2+} alone	With Ca^{2+} plus thrombin	With Ca^{2+} plus GTP
None	13 $\pm$ 2	55 $\pm$ 2	48 $\pm$ 3
Cyclic GMP	26 $\pm$ 1	82 $\pm$ 2	29 $\pm$ 5
8-Bromocyclic GMP	6 $\pm$ 1	35 $\pm$ 1	28 $\pm$ 2

Samples of a suspension of permeabilized human platelets containing granule-bound ^{14}C -serotonin were equilibrated for 15 min at 0°C with Ca^{2+} buffer giving a $[\text{Ca}^{2+}_{\text{free}}]$ of $1\ \mu\text{M}$ and, where indicated, with cyclic GMP ($10\ \mu\text{M}$), 8-bromocyclic GMP ($10\ \mu\text{M}$) and GTP ($100\ \mu\text{M}$). These samples (final volume $0.1\ \text{ml}$ containing 0.36×10^8 platelets) were then incubated, in some cases with added human thrombin ($2\ \text{U ml}^{-1}$), for 10 min at 25°C , after which the release of ^{14}C -serotonin was measured. Further details of the experimental procedures are given in refs 2 and 7. Values are the mean $\pm$ s.e.m. of three determinations in the same experiment.

centrations of GMP and GTP ($10\ \mu\text{M}$) enhance secretion of ^{14}C -serotonin from permeabilized platelets to the same extent as does cyclic GMP⁷. Knight and Scrutton¹ used 2', 3'-cyclic GMP, which had no effect, to control for nonspecific effects of the physiological cyclic GMP (3',5'-cyclic GMP), but the former compound may not be metabolized by platelets. Unlike Knight and Scrutton, we found that cyclic GMP and other guanine nucleotides enhanced secretion caused by Ca^{2+} alone, though these compounds were more effective when thrombin was also present⁷. This difference may reflect removal of endogenous guanine nucleotides by gel filtration during the preparation of our permeabilized platelets. At high concentrations (for example, $400\ \mu\text{M}$), cyclic GMP lost its stimulatory effect on secretion, suggesting the existence of a separate inhibitory action of the compound. We therefore compared the effects of low concentrations of cyclic GMP and of its metabolically stable analogue, 8-bromocyclic GMP (Table 2). In contrast to cyclic GMP, the 8-bromo compound inhibited secretion of ^{14}C -serotonin caused by either Ca^{2+} alone or Ca^{2+} plus thrombin but when secretion was induced by adding Ca^{2+} and GTP, 8-bromocyclic GMP and cyclic GMP were both inhibitory. Thus, under conditions in which the conversion of cyclic GMP to GTP can have no stimulatory effect, as must also be the case in intact platelets, an inhibitory action of this cyclic nucleotide can be demonstrated readily.

Metabolically stable analogues of GTP (for example, guanosine-5'-O-(3-thiotriphosphate) were far more effective in enhancing the Ca^{2+} sensitivity of secretion than either GTP or cyclic GMP; the effects of all these guanine nucleotides were inhibited by the GDP analogue guanosine-5'-O-(2-thiodiphosphate)^{7,8}. GTP and guanosine-5'-O-(3-thiotriphosphate) enhanced diacylglycerol formation in permeabilized platelets⁸. We have suggested^{7,8} that these observations are explained most easily if the enhancement of Ca^{2+} sensitivity by guanine nucleotides is mediated by an unidentified G-protein, similar to one of those involved in the regulation of adenylate cyclase⁹, that

stimulates the breakdown of phosphoinositides to diacylglycerol.

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KNIGHT AND SCRUTTON REPLY—Haslam and Davidson (ref. 1 and above) have provided a reasonable interpretation of the data presented previously by ourselves² and others^{3,4}. We have confirmed their finding that in permeabilized platelets GTP enhances the sensitivity of ^{14}C -serotonin secretion to Ca^{2+} in the presence of thrombin and have found that, except at high concentration ($0.1\ \text{mM}$), GTP and cyclic GMP show identical dose-response curves for this effect. The time course of secretion activated by these two nucleotides was also not significantly different. Furthermore, addition of a non-saturating concentration of GTP ($0.01\ \text{mM}$) selectively abolished the stimulatory effect of cyclic GMP on ^{14}C -serotonin secretion in the presence of thrombin plus $0.4\ \mu\text{M}$ Ca^{2+} (Fig. 1) but had no effect on the inhibition observed in the permeabilized preparation at high concentrations ($>0.1\ \text{mM}$) of cyclic GMP. An additive response to GTP plus cyclic GMP was not observed, as would be expected if separate sites of action were involved. In view of the extensive metabolism of cyclic GMP demonstrated by Haslam and Davidson (ref. 1 and the above), the concentrations required for the inhibition shown in Fig. 1 are probably not representative of those

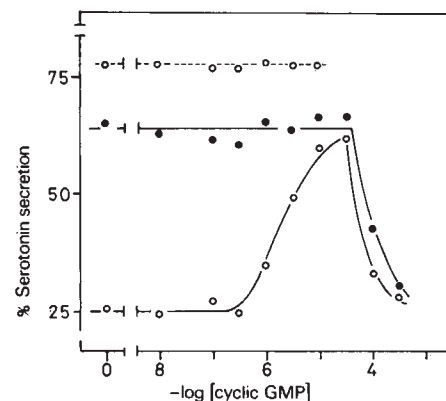


Fig. 1 Effect of 0 (○—○), 0.01 (●—●) and 0.1 (○—○) mM GTP on secretion of ^{14}C -serotonin from permeabilized human platelets induced by cyclic GMP in the presence of $0.3\ \text{U ml}^{-1}$ thrombin and $0.4\ \mu\text{M}$ Ca^{2+} . Platelets were prepared, suspended in a glycine-based buffer, pH 6.6 containing $1\ \text{mM}$ Mg-ATP^{2-} and $1\ \text{mM}$ EGTA and rendered permeable as described previously². The permeabilized suspension was then incubated at 20°C for 15-20 min with $1\ \mu\text{M}$ isobutyl methylxanthine and the indicated concentrations of cyclic GMP in the absence or presence of GTP (0.01 or 0.1 mM). Aliquots of this suspension were challenged with $0.3\ \text{U ml}^{-1}$ thrombin and $13\ \text{mM}$ Ca-EGTA , corresponding to $\sim 0.4\ \mu\text{M}$ Ca^{2+} , for 5 min at 20°C then the cells were removed by centrifugation at $8,000\ \text{g}$ for 2 min. The ^{14}C content of the supernatant fraction was estimated and expressed as a percentage of the total ^{14}C -serotonin content as described previously³.

required for an inhibitory response in the intact cell.

Although we have no evidence to contradict Haslam and Davidson's conclusions, we find it surprising that such rapid and complete metabolism of cyclic GMP to GDP and GTP occurs in the permeabilized platelet preparation and that this conversion is insensitive to an inhibitor of cyclic nucleotide phosphodiesterase at concentrations in excess of those required to prevent hydrolysis of cyclic AMP⁵. These findings imply that guanine nucleotide metabolism is more highly organized in this cell than has been suspected.

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More of the same, please

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Evolutionary Theory: Paths into the Future.

Edited by J.W. Pollard.

Wiley: 1984. Pp.271. £21.50, \$37.95.

Beyond Neo-Darwinism: An Introduction to the New Evolutionary Paradigm.

Edited by Mae-Wan Ho and P.T. Saunders.

Academic: 1984. Pp.376. £25, \$40.

WHAT WILL the next stage be for the theory of biological evolution? The contributors to these two ostensibly futuristic miscellanies only really agree on what it will not, or at least should not, be. They hope it will have as little as possible to do with the theory of natural selection. The theory is not to be dealt with by argument: it is to be wilfully ignored. In the words of Ho and Saunders's introduction to their own book (H&S)*, natural selection will be "relegated here to the [explanation of] last resort".

The relegation has been accomplished so successfully that most of the authors seem to have forgotten that the theory of natural selection ever even existed. Ho and Saunders (P) peremptorily dismiss the entire Darwinian literature on adaptation with the casual remark that those thinkers merely "cited [complex adaptations] as examples of the great power of natural selection", and after that "very little remains to be said". Elizabeth Vrba (H&S), in a similar manner, declares that "differential net rates of increase in numbers of species in monophyletic groups has received almost no consideration" from previous writers — which is a most singular description of the literature on adaptive radiation. And when Brian Goodwin (P and H&S) concludes a gliding discussion of ambiguous and slippery abstractions with the statement that the theory of evolution contains "no general principles of biological organization because all has been explained away in terms of inheritance and selection" my head swims. For what are the facts of genetics, and the explanation of homology and convergence by natural selection, if not "general principles of biological organization"? And what, for that matter (given that we are proposing and not testing hypotheses) is the difference between explaining something and explaining it away, unless the former is a hypothesis one likes, and the latter a hypothesis one would prefer people to be quiet about?

However that may be, as this post-Darwinian *avant-garde* are determined to ignore natural selection they clearly have need of something to put in its place. That is the main concern of both books. Various discoveries, they suggest, may be made in

molecular biology, in palaeontology, in classification, and especially in embryology, which may fulfil that need. A similar set of authors covers much the same ground for Pollard and for Ho and Saunders (although Pollard's book is the best of the two). Janvier (P) gives a clear introduction to cladism, marred only by his attention to the character defects (authoritarianism etc.) rather than the reasoning powers of its critics; but Nelson and Platnick's cladistic essay in H&S, I suspect, will be intelligible only to experienced readers — who else could be expected to know that, when they say things like "Darwinism ... is, in short, a theory that has been put to the test and found false" they actually *mean* that ancestral species are tricky to represent in cladistic classifications? Nostalgic Lamarckism is discussed by Steele *et al.* (P), Pollard (H&S) and Ho (H&S). Steele *et al.* conclude that the evidence is "in support of" Steele's own somatic selection hypothesis; and they do not accept Brent *et al.*'s experimental contradiction of it (*Nature* 295, 242; 1982). Brent *et al.*'s result, they say, "may in part be due to the *in vitro* CML assay employed", which is the kind of remark I need immunological help with.

In the final chapter slot, Popper (P) on "evolutionary epistemology" is probably of broader interest than Margaret Boden on artificial selection and biological reductionism and C. Sunha's "socio-naturalistic approach to human development" (both in H&S). Pollard's book, moreover, opens with a sensible chapter by Riddiford and Penny, on such subjects as whether natural selection is a tautology. Their patient review is probably made up of too familiar stuff to interest Darwinian readers; but it is still a source of great comfort. How reassuring it is to think that all those authors who cannot find anything to choose between divine creation and natural selection are going to receive a refutation of their views bound into their complimentary copies! I recommend it to them.

In both books, embryology is the main subject. Embryology potentially has much to offer to the theory of evolution, and even the most sullen Darwinian stick-in-the-mud would endorse Goodwin's (P) conclusion that "in order to understand evolution we must understand develop-

mental mechanics" — so long as it is not construed too exactly. The heritable changes responsible for the evolution of a dinosaur's forelimb into a bird's wing must have exerted their effects by means of changes in embryonic development: the transformation must have been developmental. Therefore, if we knew how development took place, we might be better placed to find out more about the course of evolution, and the kinds of mutations needed to effect it. I object only to being told that this conclusion is new, or that it topples some wretched orthodoxy: it is old hat, and none the worse for that.

Embryology might also be useful in another, related way. It might help us to understand the range of possible variation of a trait. For many traits, we know something about how natural selection would act on them, but rather less about their range of variation. In the absence of better knowledge, we have to make assumptions; but obviously they need to be substantiated. Our assumptions, it is true, do go beyond the facts; but it is an absurd exaggeration of Ho and Saunders (H&S) to say that "the neo-Darwinian concept of random variation carries with it the major fallacy that everything conceivable is possible". It does nothing of the sort. No such assumption is necessary, nor has it ever been made. Natural selection can still work if the range of variation is limited. Ho and Saunders, however, appear to deny this. In their new "paradigm", they suppose the array of forms in nature to consist only of such variations as have occurred, natural selection not having chosen among them. Thus: "whether or not a particular feature appears in evolution largely depends not on its relative fitness once it occurs, but on the probability that it ever occurs at all". This really is different from neo-Darwinism; but it is a familiar argument, rejected (or "largely" rejected) by neo-Darwinians because it (largely) does not explain how organisms could be adapted for life.

Goodwin makes a better, if smaller embryological point. He explains it in terms of limb loss in evolution; but the point is general. Biologists have often asked which particular digits have been lost from reduced limbs, and which remain. But if digits develop out of morphogenetic waves, controlled by Turing-like field equations, and a pentadactyl limb evolves into a tetradactyl one when a standing wave changes from a five-peaked to a four-peaked condition, it could be nonsense to ask which digit had been lost: the four digits do not really correspond to any four out of the original five. If, with Goodwin, we can take it for granted that limbs do indeed develop in this way (and he says it is "amply demonstrated in developmental biology"), the point should be well taken. At all events it is a curious conceptual possibility.

Goodwin's suggestion easily fits in with

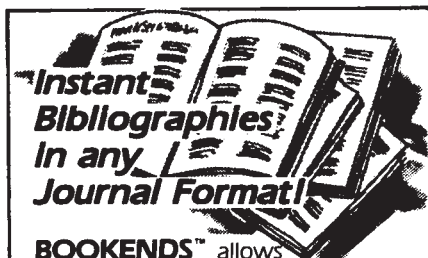
* H&S stands for a chapter in Ho and Saunders, P for one in Pollard; the two books share several contributors in common.

what is already thought about evolution. It is a modest contribution, but a real one. He, however, would see it otherwise. Like his fellow authors he is resolved on revolution. He believes that his work exemplifies a new "generative paradigm" for biology, which ought to replace the philosophically less satisfactory evolutionary "paradigm" that biologists persist in deceiving themselves with. Now, I do not think biology even has "a" paradigm; but I do not want to argue about that: what I do deny is that Goodwin's work demands any deep changes in the theory of evolution.

In general, these volumes completely fail to show that natural selection should be relegated to the explanation of "last resort". Not all the ideas discussed are wrong, or unimportant: but those that are probably correct are already incorporated in the Darwinian theory, or can be added to it without conceptual difficulty; and those that are not, have for that reason already been rejected. Darwinism is not as vacant, nor as silly, as these authors portray it. I too hope to spend my declining years thinking about evolution. But I still expect to make frequent resort to Mr Darwin's theory, which has survived over a century of criticism: to his theory of natural selection. After all, what else do we have? □

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Shadows on the wall

P.W. Atkins

In Search of Schrödinger's Cat: Quantum Physics and Reality.

By John Gribbin.

Wildwood House/Corgi/Bantam: 1984.

Pp.302. Hbk £12.50; pbk £2.95, \$8.95.

Quantum Questions: Mystical Writings of the World's Great Physicists.

Edited by Ken Wilber.

Shambhala, PO Box 271, Boulder, Colorado/Routledge & Kegan Paul, London: 1984. Pp.208. Pbk \$8.95, £8.95.

THESE books confront us with two totally different yet intellectually entwined approaches to that most extraordinary of this century's revelations. Gribbin's popularization of quantum theory I approached with sympathy; Wilber's collection of essays with something akin to scorn.

In Search of Schrödinger's Cat is the more straightforward book. It is one of a small number, none of them particularly successful, which attempt to convey the content of quantum theory to a lay audience. That audience is generally presumed to expect bewilderment, the folk-appreciation of quantum theory, and generally it gets it. Popularizations begin with the precept that quantum theory has overthrown our most fundamental expectations of the world, and has replaced them by deep perplexities. Gribbin shares with his readers his astonishment and delight that so much oddness may be taking place around us, and successfully conveys his opinion that deep down the world is mysterious beyond our dreams. All this he does with an admirable desire to convey insight and wonder. However, the execution is uncontrolled, and for many pages he wanders off into the mechanisms of lasers and semiconductors. These excursions are intrusive, and the book would have had more impact without them.

Be that as it may, I must confess that I do not approve of the impact the book might have had. Gribbin favours awe. That certainly makes for better journalism, but in the later stages of his book, after we have been saturated with astonishment, he unfurls his true colours and flies the flag of many worlds. This minority interpretation of quantum theory is plainly a science fiction writer's joy machine: it is the opium of the physicist, and being so outlandishly profligate (resembling the hypothesis of God) its acceptance seems to me to abnegate the scientist's duty to seek truth in simplicity.

Gribbin's book is a child of the public wonderment of the discoverers of quantum theory, and his awe is fuelled by theirs; for since the founders of the subject were bewildered it is hardly surprising that their interpreters convey a sense of stupefaction. I, however, prefer to think of the quantum fathers as being so steeped in their classical

expectations that they could never loosen their minds to accept the greater simplicity their discoveries implied. Even Heisenberg, who is represented in Wilber's collection of essays, seems to me to have misunderstood the uncertainty principle by interpreting it as limiting our ability to know the present, rather than appreciating that it constrains the meaning of "complete description", and not our ability to know it. Classical physics, quantum theory shows, is *overcomplete*, and attempts to impose descriptions that are too complete for reality to bear; quantum weirdness stems from our insistence on answers to classically conditioned, meaningless questions.

But *Quantum Questions* goes beyond the misconceptions that plagued the founding fathers. It is a collection of their essays in which they reflect on mysticism, technically interpreted to mean the direct discernment of reality beyond the image, and in particular the experience of the reality beneath the mathematics. There is, of course, a narrow line between hallucinations, in which the divine is purportedly directly experienced, and the kind of comprehension being groped for by the quantum physicists as recorded in these informal essays. They could dimly see that the homomorphism of mathematics and the world, the fact that mathematics works and is our key to the exploration of the workings and structure of the world, must entail something much deeper than at first might appear. They variously muse and strive in these essays to identify the substance beyond the mathematical shadows on the wall. For what it is worth, I am sure they are right, and that the hand-in-glove fit of mathematics and "reality" must be more than a coincidence. I personally suspect that it reveals a consonance of the logical structure of mathematics and the structure of spacetime, and that one is in some sense in resonance with the other.

I emerged from Wilber's collection, and his charmingly seductive introduction, in some sympathy with these night-thoughts. Of course, there is no resolution of the central question, and there is a great deal of foggy twaddle peddled by the pessimistic: Schrödinger views science as silent on the world near the heart; Heisenberg remarks that one cannot think about ethical problems rationally, and de Broglie seems unduly contaminated by the deep pessimism of Bergson, who was dangerously influential yet failed to appreciate, as some still do, the extraordinary and probably limitless power of science. Yet, among the twaddle there are thoughts worth thinking. Those with a mind attuned to the deeper problems of the world will find little satisfaction from this volume, but they should take pleasure from much, and generally wholesome, provocation. □

P.W. Atkins is a Fellow of Lincoln College and University Lecturer in Physical Chemistry at the University of Oxford.

Eye to the rosy lens

Dorothy Nelkin

Science and Scientific Researchers in Modern Society.

By John P. Dickinson.

UNESCO: 1984. Pp.254. FF90.

JOHN Dickinson is an organic chemist who has specialized in biomedical research in clinical laboratories. At present he is an executive in a pharmaceutical firm, with responsibilities for science administration. It is from this diverse perspective that he writes about the role of science and technology in modern society. He begins by expressing distress about what he views as growing public antipathy to science, his end purpose being to demonstrate that in its methods and ethics, as well as its specific contributions, science is an important and positive agent of progress.

Dickinson describes the distinctive features of scientific research, distinguishing it from other data-gathering activities. Tracing the scientific method through the formulation of theories and hypotheses, and the testing of hypotheses by experiment, he argues that it is "the leaven of disciplined imagination which imparts to scientific research a special place among other intellectual activities". He also considers the relationship between fundamental and applied research, drawing attention to the symbiotic and synergistic relationship between science and technology, and the importance of this relationship to the social contribution of science.

With this as background, Dickinson turns to the education and socialization of scientists, suggesting how they are prepared to deal critically and creatively with the multi-faceted and often shifting character of problems. He then describes the social environment of research, career and mobilization patterns, job satisfaction and the question of public recognition.

In the book the scientist is examined as both a professional and a citizen. As professional scientists are faced with several responsibilities that are sometimes in conflict; for example, their responsibility to "an invisible college" of peers may sometimes conflict with their responsibility for the possible use or abuse of their work. In looking at the scientist as citizen the focus is upon the researcher's responsibility to society, and Dickinson notes the recent shift from the traditional apolitical stance as scientists have increasingly become engaged in controversial issues.

This book wades into most of the major issues in "science and society", the author himself emerging as a profound optimist. Dickinson sees science and technology as the solution to many problems, ranging from the depletion of non-renewable resources to cancer: science is "the potent fuel of change and progress", to be viewed

"not only as promoting intellectual freedom generally, but also as enriching culture in the broadest sense". It is an activity that is basic to the construction of "world equity and peace". Thus he calls for scientists to "exercise greater influence upon political life" at all levels.

It is with this attitude that Dickinson returns to his worries over science as a scapegoat. He sees anti-science sentiments as dangerous, threatening and widespread. Yet, violating his own description of how scientists work, he offers no supporting evidence for the assertion. In fact, the evidence available from surveys, and suggested by both public interest in popular science and the tone of most media coverage, is quite otherwise. Dickinson, however, is not alone in his view. Many scientists tend to translate public criticism of certain technologies such as nuclear power, or concern over specific scientific procedures into wholesale rejection of the

entire scientific and technological enterprise. I find this curious. Why do scientists expect to be loved? After all, lawyers, doctors, journalists and businessmen are routinely subject to public criticism. Should scientists be exempt? For some reason, no other profession, except perhaps the clergy, expects to be so revered.

Dickinson has painted a helpful and broadly based picture of the scientific community in its many and undeniably important roles. But it is a picture that is more likely to increase awe and overexpectation than to lessen the gap between science and the public. I wish the author had used his material to create a warmer portrait of scientists, less as saviours than as real people engaged, as so many others, in a useful and necessary human enterprise.

Dorothy Nelkin is a Professor in the Cornell University Program on Science, Technology and Society and the Department of Sociology.

Biology by numbers

George F. Oster

Modeling Dynamic Phenomena in Molecular and Cellular Biology.

By Lee A. Segel.

Cambridge University Press: 1984. Pp. 300. Hbk £25, \$49.50; pbk £8.95, \$12.95.

PROFESSOR Segel heads the Applied Mathematics Department at the Weizmann Institute, and is one of the leading figures in that shady profession known as biomathematics. This book emerged from lecture notes for a course in mathematical modelling which he has given for several years. Some biologists may still wonder at the demand for such a course. Aside from the "physics envy" that Joel Cohen claims is the curse of biologists, isn't biology still an essentially empirical science? Aren't courses in mathematical biology merely pandering to idle physicists' and mathematicians' lust for "relevance"? Well, perhaps, but I feel that perusal of this book by any but the most recalcitrant empiricist may thaw that viewpoint.

Professor Segel has striven hard — and succeeded quite well — to select topics of genuine biological interest, and to present their mathematical underpinnings in as clear and understandable a way as one will find anywhere. After all, what is mathematics but a set of rules for logical thinking? And what biologist would shrink from logic? But of course, the issue is not that simple. Biology is, after all, a largely inductive enterprise, and many eons away from the physicists' clean mathematical formulations. The real test of theory comes when the biologist asks: what does mathematics tell me that I didn't know already? Or, what experiments could it suggest that I wouldn't have thought of myself? To the former question a theorist

might reply that talk is cheap. It is not sufficient merely to say that something is so: that such and such a conclusion follows from this assumption and that. Only a calculation based on a clearly formulated model can entitle one to draw firm conclusions. Nonsense, replies the biologist. More things are true than you can prove by theorems. And so the argument goes. Finally, I suppose, it is a matter of taste whether one finds mathematical models enlightening or confusing. Despite Darwin's envy of the mathematician's "extra sense", his theory did not suffer much for lack of it.

Professor Segel seems to have had this debate in mind, for he takes care to discuss just what the role of mathematics is in each of the topics he covers. Moreover, he eschews — wisely, I feel — the practice of "general theorizing". He bases each chapter on a particular empirical problem, and builds models which address the issues the experiments raise. The level of mathematics progresses from very elementary in the early chapters to quite advanced in the later ones. However, mathematically untrained readers may never detect the altitude, for Professor Segel has the gift of making advanced notions appear simple. The book is crowned by a collection of appendices which provide sufficient background mathematics that most readers will not have to consult outside sources.

Overall, Professor Segel has done an admirable job of presenting a broad range of mathematical models and techniques at a level suitable for upper division students in the life sciences. For anyone with the slightest interest in modelling biological situations, there can hardly be a better elementary text. □

George F. Oster is Professor of Biophysics, Entomology and Zoology at the University of California, Berkeley.

Heisenberg: the man and his physics

Nicholas Kemmer

Werner Heisenberg: Collected Works, Series B.

Edited by Walter Blum, Hans-Peter Dürr and Helmut Rechenberg.

Springer-Verlag: 1984. Pp.937. DM108, \$38.

Inner Exile: Recollections of a Life with Werner Heisenberg.

By Elisabeth Heisenberg.

Birkhäuser: 1984. Pp.170. SwFr. 38.

BY THE time Werner Heisenberg had reached his twenty-fifth birthday he was already a leading figure in the world of theoretical physics. His greatest achievements date from his young days, but he continued to do most important work in a great variety of fields of physics for many years and was still working on deep problems until quite close to his death. He was the teacher of numerous top-rank physicists and a highly skilled expositor in lectures, books and articles, not only within physics but also on many other subjects, among which philosophy had an important place.

Editing his collection of works is an enormous task, not yet completed, and the present volume is the first of several. It forms the whole of Series B, to be sandwiched between the multi-volume Series A (primary scientific publications, also published by Springer) and Series C (works outside physics, published by R. Piper, Munich). In this volume works of an expository nature have been brought together: books, articles in symposium volumes and journals, public lectures, right down to remarks in conference discussions. Every piece of material is reproduced as it first appeared (in some cases in typescript with handwritten equations) except that everything originally of large format has been reduced, sometimes to the detriment of legibility. Items appear in German, English, French and, in one case, Dutch.

For a non-specialist this volume provides an easier survey of the vast range of scientific knowledge we owe to Heisenberg than will Series A. In the first half or so, the branches of physics covered are remarkably varied and reveal interesting links between apparently unrelated fields. However in the latter half almost all the material is concentrated on one topic, Heisenberg's Unified Theory of Elementary Particles which he single-mindedly started to develop 30 years ago in collaboration with a small number of colleagues. This work continued parallel with and, up to a point, in full cognizance of the rapidly changing knowledge being accumulated elsewhere, but it has not as yet been accepted as valid by physicists in general. A very

relevant insight into the situation as it stood in 1958 is provided on p. 563 by Heisenberg's report on his theory and the ensuing strongly critical discussion led by Wolfgang Pauli.

Brief mention should also be made of the few pages in this volume taken from post-war publications (produced under American auspices) about the lines along which work in Germany, aimed at the utilization of fission energy, had proceeded under Heisenberg's direction. As references to details of that research are exclusively to unpublished wartime reports, the information provided is rather limited. However what we do learn of the story links up with the second book reviewed here.

The German original of *Inner Exile* was presented to me by a friend. I could not resist reading it immediately from cover to cover. In it Elisabeth Heisenberg reveals

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

At home — Werner Heisenberg in 1947, at Göttingen, where after the war he became director of the Max Planck Institute for Physics.

herself as a gifted and sensitive writer with a wonderful skill in the use of her native language. The translators could not have been expected to match the rich quality of the original prose, but I did not find any serious faults in their work, except for one glaring mistranslation that touches on something of importance. In an early passage telling of her husband's boyhood, the author quotes a word used by a colleague to describe occasional bouts of hot temper. This word, "Blutrausch", is rendered as "bloodlust". The passage indicates that Mrs Heisenberg does not depict her husband as a saint, but the English word would imply a streak in his character that is diametrically opposite to what she would wish to convey. This book need not be seen as putting to rest all controversy about Heisenberg's personality, but it is a pity that this small word should slip in, fitting as it does the vocabulary of his meanest detractors.

The aim of the book is to relate how Heisenberg, after much conflict, decided not to emigrate from Germany after Hitler's rise, even though he detested Nazism and suffered and was endangered

personally for failing to support "German Science". We are told how and why he concluded that his place was in his home country.

I prefer the German title of the book (*Das politische Leben eines Unpolitischen*) to the English one with its slight element of pre-judgement which the book otherwise does not demand of the reader. The excellent introductory chapter by Victor Weisskopf expresses most sympathetically how a sensitive and complex personality such as Heisenberg's would suffer under the pressures upon him. To an infinitely lesser extent, I experienced them myself. Nevertheless I do not think that the book will be seen as the final word on this piece of history. After all, today — 40 years after the Hitler nightmare ended — I think I am not alone, having re-built most happy personal contacts in Germany, in having buried deep inside me residues of old sores and bitterness that will not be wiped away. They re-emerge, for instance, when I am reminded of the way a formerly close colleague of Heisenberg's returned from a post-war reunion in deep distress — a gulf remained unbridged.

In the book we are told of a war-time meeting between Heisenberg and Bohr, and of a disastrous failure of communication between them. A brief account of the same event is given by Heisenberg in his autobiographical work *Der Teil und das Ganze* (published by Allen and Unwin in 1971 as *Physics and Beyond* — another distorted title!). The greatest part of that book consists of reconstructions in dialogue form of thoughts shared, developed and deepened by the author with Bohr, Pauli, Dirac and many others. As a way of conveying these ideas the presentation is attractive and absorbing; nowhere is it claimed that the mumbling Bohr, the laconic Dirac or the sharp-tongued Pauli could actually have spoken like that — nor written the same story. I wish I could have seen a version by Niels Bohr of that war-time meeting with Heisenberg.

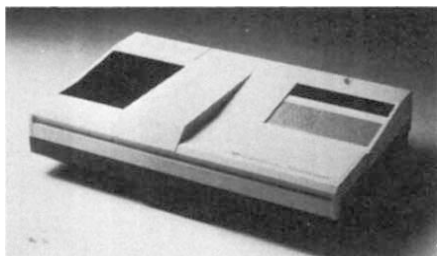
These thoughts came to me when I was reading the last few pages of Elisabeth Heisenberg's book. There she mentions "bitter scientific quarrels" between Heisenberg and Pauli over the Unified Theory. After Pauli's death they were touched on by Heisenberg in a short memorial lecture given at a meeting in Trieste (unfortunately not included in the published proceedings of that event). There he linked Pauli's abrupt turning away from his own ideas to the onset of the latter's terminal illness. This view brought about an emphatic contradiction from my dear teacher, the late Gregor Wentzel. It is not recorded, but I can vouch for it. This is but a minor point — yet it is a fragment of historical disagreement that I think is worth recording. □

Nicholas Kemmer is Emeritus Professor of Mathematical Physics at the University of Edinburgh.

Microbiologists choose Las Vegas

The American Society for Microbiology meets next week in Las Vegas. Here we describe some of the equipment on view at the exhibition and other new wares for microbiology.

● A new automatic microbiological media preparation system is available from Jouan, Inc. Two variations are offered. Model SH05 C is capable of preparing batches from 0.5 to 5 litres per run and model SH10 C has a 1.5 to 10 litre capacity. Operation is simple, a measured amount of dehydrated medium and water is placed into pressure vessels, and the rest is automatic. The electronic programmer can be used to set sterilization temperatures and time as well as dispensing temperature. *Circle No.100 on Reader Service Card.*



Automated mutagenicity tests by Labsystems.

● Mutascreen, developed and produced by Labsystems, is an automated assay and analyser system for bacterial mutagenicity. The test is based on the detection of point mutations in *Salmonella typhimurium* as used in the Ames test. Samples, bacteria and the metabolic activation system are automatically dispensed by the analyser, the bacterial cells mutate and grow in liquid medium, and their auxotrophic and prototrophic growth is monitored photometrically. The photometric system is based on vertical measurement so that all cells are within the reach of the light beam, and the sedimentation of the cells does not affect the absorbance of the samples. All data on the bacterial growth patterns are stored in a microcomputer.

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● Cambridge Technology, Inc. has published a new two-page brochure on the accessories available for the company's PHD cell harvester. New accessories include a quick disconnect system which allows different harvest heads to be changed quickly and easily, and a four-port wash manifold which permits as many as four different wash fluids to be used without changing tubing connections. Other accessories such as a flow control valve, supernatant collection system, special harvest heads as well as vial trays and pre-cut filter paper are also described.

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These notes are based on information provided by the manufacturers. For further details circle the appropriate numbers on the Reader Service Card bound inside the journal.



TCS dehydrated media.

● Tissue Culture Services now offers a range of the 'Top Thirty' dehydrated media for microbiology. Available in 500 g tubs, the new media complement the existing range of ready-poured plates, broths, slopes and blood products for the microbiological laboratory. As an added service, TCS can also supply customer-specific media.

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● The Mouse bench-top bioreactor system for fermentation and cell culture is a bench-top mainframe featuring an on-board computer with 8- to 32-bit data handling capability, which can control and monitor up to 21 parameters simultaneously. All values are simultaneously displayed on a high-resolution CRT. Digital control of the mainframe's 1/3 HP, high-torque drive motor provides precise control of agitation at speeds ranging from under 20 r.p.m. to 2,000 r.p.m. Four interchangeable top drive and airlift vessels attach quickly and easily without tools. The control and monitoring of temperature, air flow and agitation speed are standard. The Mouse is manufactured by Queue Systems, Inc.

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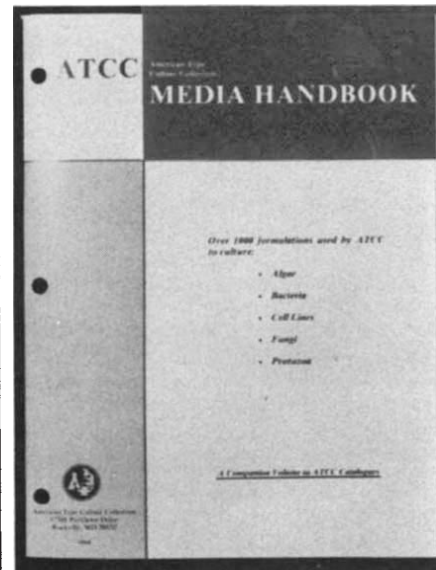
The Vibra-Cell cell disruptor.

● At the ASM meeting Sonics & Materials, Inc. is introducing a new low-power ultrasonic processor. The Vibra-Cell can disrupt cells, bacteria, spores, or tissues, prepare an emulsion down to 1/100 of a micrometre and homogenize liquids. In addition, the Vibra-Cell can be used to extract sera, toxins, enzymes and viruses from organic sources.

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● Elkay is introducing three new products of special interest to microbiologists at the ASM Convention in Las Vegas (Booth 635-636). Elkay Bioloops are designed to make media inoculation safer and simpler. Bioloops are sterile disposable inoculating loops and needles which do not need flaming before use. There are three different models, all available individually wrapped and in 10-piece tray packs. There is a comprehensive choice of sizes in Elkay's new sterile culture tube line, ranging from 10 x 75 to 17 x 100 mm in optically clear polystyrene and chemically resistant polypropylene. These tubes are available individually wrapped and in easy-to-store tray packs. For centrifugation, Elkay introduces 15 and 50 ml. capacity sterile and non-sterile disposable tubes. These come with leak-tight screwcaps, moulded-in graduations and optional Styrofoam rack pack. Every microbiologist visiting the Elkay booth at Las Vegas will receive a free sample of the new Elkay Ezee-Write Labmarker which can be used to mark virtually any surface, wet or dry.

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● A new laboratory guide for use in stock culture maintenance is now available free from the American Type Culture Collection. The 98-page handbook contains over 1,000 media formulations used at ATCC to propagate strains of algae, bacteria, cell lines, fungi and protozoa. Step-by-step instructions are given for media preparation. Entries are indexed both alphabetically with medium name and ATCC medium number, and by species name used in ATCC catalogues and the medium or media used at ATCC to culture each organism.

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● A rapid slide identification kit for *Streptococcus pneumoniae*, giving results in as little as 2 minutes, is now available from BioMerieux. The kit identifies all the 83 serotypes from either liquid (blood) or plate cultures. Also included are both positive and negative controls with enough reagents for 50 tests. Also new from BioMerieux is Staphyslide, a slide agglutination test for the rapid identification of 98% of *Staphylococcus aureus* strains. Staphyslide is an improved coagulase method using fibrinogen sensitized red blood cells. Each Staphyslide kit contains sufficient reagents for at least 50 tests and includes a negative control. The only other



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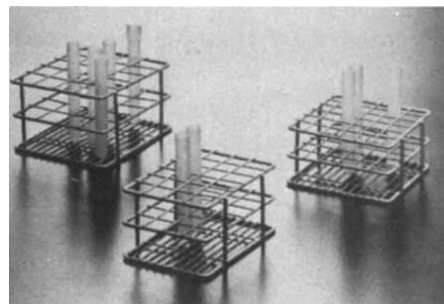
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● Optomax, Inc. has introduced a television scanning image analyser specifically aimed at biology/microbiology applications. The 40-10 System is microprocessor based and incorporates applications software as standard. Suitable for use either directly with a microscope or with the Optomax Macro Stand, applications include chemotaxis assays, inhibition zone diameter measurements, autoradiograph grain counting, colony and plaque counting, fluorescent bacteria counting and cell clone counting. Data are presented on a front panel display and hard copy is provided by the built-in printer. Options for 40-10 include interfaces to external computers, automatic Petri dish rotation (for inhibition zone analysis) and autofocus.

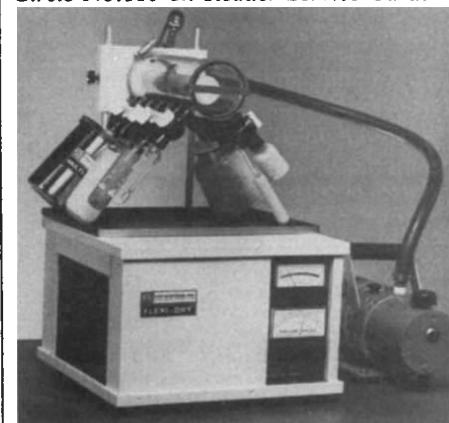
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Test tube racks for smaller spaces.

● If half-full test tube racks are cluttering your refrigerators and benchtops, the new space-saving 'half racks' from Bel-Art Products may solve your problems. A new addition to Bel-Art's Poxgrid line of epoxy-coated wire racks, the half racks are resistant to most chemicals, and are autoclavable. Available in green, blue and orange, there are three sizes of rack holding 10-13, 15-16, and 18-20mm tubes in 36, 24, and 20 places respectively.

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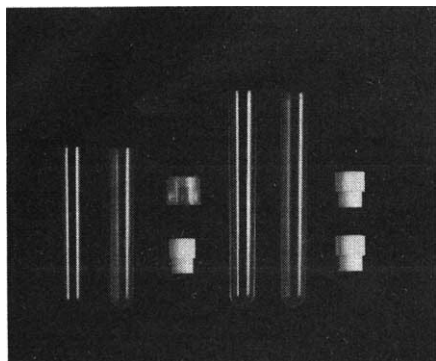


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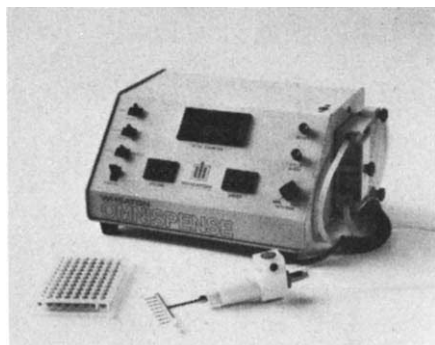
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Aerobic and anaerobic cultures are possible in these Bio-Rad culture tubes.

● The new 12-mm dual position cap for Bio-Rad culture tubes is really two closures in one. It provides an overcap which fits loosely outside the top of the culture tube to protect the contents from dust. The sides are held away from the surface of the tube by vertical ribs, which allow air to enter so the tube may be used for aerobic culturing. The cap also contains an internal plug-type closure, which only comes into use when the cap is pressed tightly in place. When this is done the top edge of the tube engages a sealing groove in the cap to form an airtight seal, so the tube may be used for anaerobic applications. The internal ribs in the Bio-Rad culture tubes do not abrade, minimizing the risk of contaminating with 'shavings'.

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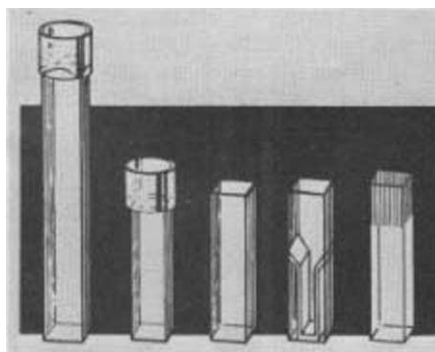
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Cleaners and disinfectants available through Forma.

● A new range of disinfectants for laboratory cleaning is now being distributed by Forma Scientific. Amphy Spray, O-Syl and Roccal II are ideal for general laboratory cleaning and disinfecting. Amphy Spray has been proven effective in tests against staphylococci, fungi, coliform and many other microorganisms. O-Syl combines disinfectant and detergent to destroy a wide variety of pathogenic organisms. Roccal II inhibits alga growth in water-jacket incubators in addition to sanitizing lab surfaces. Use of such disinfectants reduces cross-contamination. Test results are available from Forma Scientific.

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Sarstedt disposable cuvettes.

● A complete line of precision moulded disposable cuvettes with excellent transmission characteristics is available from Sarstedt, Inc. These cuvettes allow assays to be performed directly in cuvettes and eliminate the need for test tubes and transfer to glass cuvettes. Included in the line are polystyrene and acrylic two- and four-sided cuvettes, with and without stoppers; semi-micro cuvettes for small samples; and a special cuvette for the Leitz Photometer, LKB Lumenometer, and other popular instruments. Cuvettes are offered in polystyrene, usable to 340nm and acrylic for use down to 280nm. Colour-coded stoppers are also offered. Light paths are 1cm.

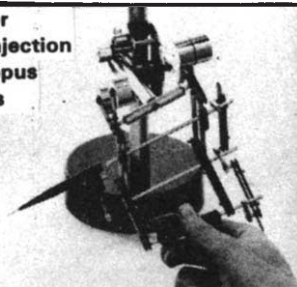
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DNA synthesis by Cruachem

A PHOTOGRAPH of the new Cruachem system capable of four parallel DNA syntheses was incorrectly captioned as a DNA 'separator' on page 509 of the 7 February issue of *Nature*. For full details of Cruachem's range of DNA synthesizers circle number 116.

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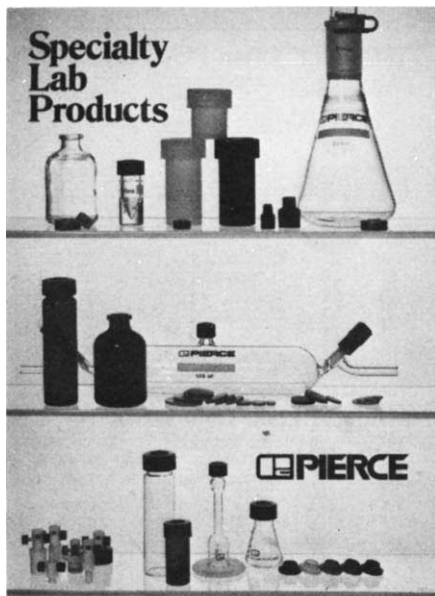
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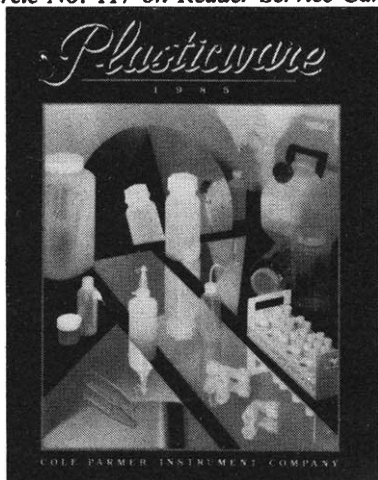
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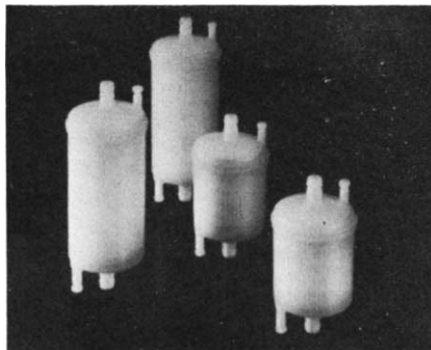


● Pierce Chemical Company has issued a new brochure entitled "Specialty Lab Products" covering sample collection, handling and storage products. Featured are: Tuf-Tainer Teflon vials, Mininert valves, Teflon/rubber laminated and Tuf-Bond Teflon/silicone disks, Reacti-Vials, screw cap septum vials and Hypo-Vials. Also included are specialty glassware products including tilt dispensers, screw cap septum volumetric flasks and gas sampling tubes. *Circle No. 117 on Reader Service Card.*



● The 1985 Cole-Parmer Plasticware catalogue is a 96-page book including a guide to the structure and properties of resins, a chart of the chemical resistance of plastic resins, and a guide to the use and care of plasticware. New items featured in the catalogue include a line of autoclavable beakers made from Haler, a new fluoropolymer resin resistant to a variety of corrosive chemicals and organic solvents; a low-priced line of autoclavable wash bottles; a line of polystyrene cabinets that can be expanded to fit particular needs and which offer four models with different drawer configurations; and a second assortment of the popular KECK clamp kits that features three of each of the nine colour-coded sizes available in the line. *Circle No. 118 on Reader Service Card.*

● Vanguard International of New Jersey, a US distributor for products manufactured by Sartorius GmbH, now offers Sartobran and Sartofluor capsules. The disposable pleated filter units are ideal for small to medium (10-500 litres) filtration. The capsules are autoclavable and are available in one and two square foot versions with pore sizes ranging from 0.2 to 1.2 micrometres. The hydrophobic Sartobran capsule has an integrally pleated prefilter, cellulose acetate pre-membrane



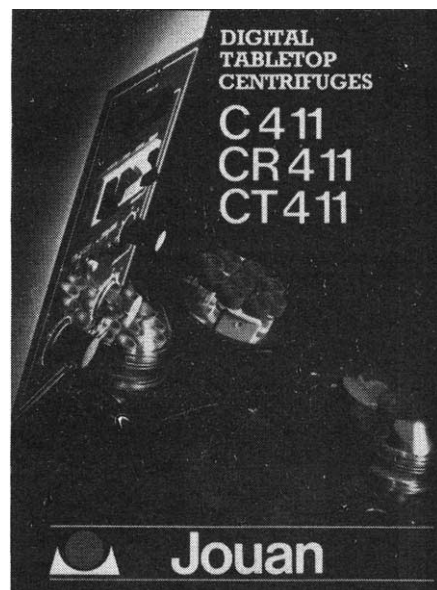
Sartorius filters — available from Vanguard International.

and cellulose acetate final membrane filter in a self contained polypropylene housing. It provides serial filtration, heat stability and low adsorption and is excellent for sterilization of tissue culture media. The hydrophobic Sartofluor is widely used as a tank vent and sterile air supply filter. *Circle No. 119 on Reader Service Card.*



The Beckman range of J-6 centrifuges.

● A new medium-priced centrifuge has been added to the Beckman Instruments J-6 range, bringing to three the number of 6,000-r.p.m. refrigerated floor models available from the company. The J-6M/E features the microprocessor controls of Beckman's top-of-the-line J-6M model, but instead of a brushless induction drive, it has the d.c. drive of the other instrument in the line, the J-6B analog model. The intermediate M/E model offers such other J-6M features as a programmable memory capable of storing up to ten programs; digital logic for precise speed, time and temperature settings; and a choice of acceleration/deceleration rates. *Circle No. 120 on Reader Service Card.*



● A new brochure from Jouan, Inc. describes the company's C411 ventilated and the CR411 refrigerated digital table-top centrifuges. The digital readouts are accurate to 10 r.p.m. for speed and 0.1° C for temperature on the refrigerated model. The C and CR411 offer high performance and are capable of speeds up to 6,000 r.p.m. generating 5,840g. They are capable of handling up to 1.18 litres per run. In addition, the C and CR411 offer variable braking control to prevent resuspension of sensitive separations as a standard feature. A full range of rotors and inserts capable of handling tubes as small as 250 microlitres or bottles as large as 450 millilitres are described in the brochure. *Circle No. 121 on Reader Service Card.*

● The 850A television camera is specifically designed for microscopy and measurement applications. The 850A provides high resolution with low noise level, and a wide selection of image tubes is available for a variety of applications. *Circle No. 122 on Reader Service Card.*

● Ten major sources of fermentation equipment failure — and how they are countered in an advanced-design open-frame fermentor — are discussed in a new brochure from New Brunswick Scientific Co. The brochure states that gross engineering errors are being made today in the design of pilot-plant and industrial fermentors. Among the areas covered are air-flow control, shaft seal reliability, control of oxygen concentration and process sterility. *Circle No. 123 on Reader Service Card.*

● A novel two-step cap, the "Cap-Lok", is a feature of the new disposable 1.5-ml and 0.6-ml microcentrifuge tubes from Robbins Scientific. The first step allows easy, one-hand, non-splash opening, but seals tightly enough for spinning and to prevent leaking. The second, tighter, Cap-Lok step provides the optimum seal for boiling, storing, or shipping. The polypropylene tube is fully autoclavable. *Circle No. 124 on Reader Service Card.*